MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF THE *S-ADENOSYLMETHIONINE DECARBOXYLASE* GENE OF *LEISHMANIA DONOVANI* AND *TRYPANOSOMA BRUCEI*

by

Jerry R. Scott

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(Professor in Charge of Thesis)

[redacted]

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(Chairman, Graduate Council)

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SUMMARY

Trypanosoma brucei (T. brucei) and *Leishmania donovani (L. donovani)* are protozoan parasites that are responsible for significant diseases of both man and his domesticated animals, making these organisms of immense clinical and economic importance. Confounding the treatment of these diseases is not only the lack of non-toxic chemotherapeutic agents, but also the development of drug resistance. The East African trypanosome is often not only refractory to the more traditional heavy metal compounds, such as the arsenical melarsoprol, but to the newer drug, difluoromethylornithine (DFMO) as well, leaving physicians with few alternatives. The situation is similar with leishmaniasis, where the pentavalent antimonial compounds have been applied therapeutically for almost 70 years and resistance to these drugs is fast becoming both a clinical and an epidemiologic problem. Furthermore, leishmaniasis has proven to be particularly difficult to control from an epidemiologic standpoint due to its multitude of animal reservoirs and the wide distribution of its insect vector, the phlebotamine sandfly. Because of the failure of more conventional control methods, there is a necessity for the development of novel and more effective chemotherapeutic agents for the treatment of the diseases caused by these organisms. As demonstrated by DFMO, the polyamine pathway has been shown to be a promising target for the design of innovative anti-parasitic drugs. DFMO is the only newly developed antiparasitic agent to reach clinical use for the treatment of African trypanosomiasis in 50 years and owes its trypanocidal action to its ability to inhibit ornithine decarboxylase, a key enzyme of the polyamine biosynthetic pathway, thereby depleting the parasite of the polyamines necessary for rapid proliferation. S-adenosylmethionine

decarboxylase (AdoMetDC) is another key regulatory enzyme of the polyamine pathway which is reportedly targeted by a number of antiparasitic and antiproliferative drugs. More specifically, it has been proposed that the enzymatic inhibition of the trypanosomal AdoMetDC enzyme by the experimental drug 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5' deoxyadenosine (MDL 73811) (MDL = Merrel Dow Laboratories) can effectively cure murine models of drug resistant African trypanosomiasis. It was our hypothesis that the molecular and biochemical characterization of the *AdoMetDC* gene and enzyme from *L. donovani* and *T. brucei* would further our understanding of the polyamine biosynthetic pathway and its regulation and thereby aid in the design of novel chemotherapeutic strategies. Because of the difficulty in obtaining sufficient amounts of enzyme to study from cultured cells, a key goal of this project was the high level recombinant expression of at least one of the parasite *AdoMetDC* genes. An additional hypothesis was that the development and characterization of an MDL 73811-resistant *L. donovani* cell line would further elucidate the mode of action of MDL 73811.

Our first aim involved the isolation of the AdoMetDC genes from *L. donovani* and *T. brucei*. To this end we attempted to identify the AdoMetDCs by screening genomic libraries from both *L. donovani* and *T. brucei* by cross-hybridization to the human *AdoMetDC* cDNA. Because of the high degree of divergence between primary structures of the parasite and human AdoMetDC proteins, this was unsuccessful. Our second approach involved the amplification of segments of both genes from their respective genomic DNAs via PCR, using degenerate oligonucleotide primers complementary to regions of high identity between the human and yeast AdoMetDC protein sequences. PCR

successfully amplified a 720 bp segment of the leishmanial *AdoMetDC* gene that was employed as a probe to isolate the entire gene from a genomic λ-GEM11 library. Sequence analysis of the leishmanial *AdoMetDC* gene revealed a single open reading frame of 1,176 bp which encoded a protein of 392 amino acids. The primary structure of the *L. donovani* AdoMetDC enzyme had 26% and 22% identity to the predicted sequences of the human and yeast enzymes, respectively. The *L. donovani* AdoMetDC is larger than the corresponding human enzyme with a predicted M_r = 44.2 kd for the immature proenzyme and M_r = 33.2 and 11.1 kd for the α- and β-subunits, respectively, of the active enzyme.

The predicted amino acid sequence of the *L. donovani AdoMetDC* was compared with the primary structures of the other eukaryotic AdoMetDCs, making it possible to redesign the oligonucleotide primers and again attempt to amplify the trypanosomal *AdoMetDC*. Although PCR still did not successfully amplify the *T. brucei AdoMetDC*, it amplified a portion of the AdoMetDC gene from the related organism, *Trypanosoma cruzi (T. cruzi)*, which is evolutionarily midway between *L. donovani* and *T. brucei*. Because the exact amino acid sequence of the *T. cruzi AdoMetDC* PCR product at the 5' sense oligonucleotide primer site was obscured by the primer itself, we amplified the mature *T. cruzi AdoMetDC* transcript from reverse transcribed mRNA using a 5' sense oligonucleotide common to all trypanosomatid mature mRNAs and a reverse primer complementary to a region of the amino acid coding portion of the *AdoMetDC* gene. The predicted amino acid sequence of the mature *T. cruzi AdoMetDC* mRNA clarified the sequence at the primer site and the added information allowed the design of PCR primers, which were ultimately successful in amplifying a 717 bp segment of the *T. brucei AdoMetDC* gene. This PCR

product was used to isolate a clone from a genomic library that encompassed an open reading frame of 1,110 bp and encoded a protein of 370 amino acids. Analysis of the predicted amino acid sequence of the *T. brucei AdoMetDC* yielded a calculated M_r = 41.7 kd for the immature proenzyme, which although 6% smaller than the *L. donovani* enzyme, is still 3.4 kd larger than the human enzyme. The subunits of the active enzyme had a predicted M_r = 31.7 and 9.7 kd for the α- and β-subunits, respectively. Alignment of the predicted amino acid sequences of the *AdoMetDC* genes revealed that the *T. brucei AdoMetDC* gene had 67% identity to the primary structure of the *L. donovani* protein and 26% and 22% identity to those of the human and yeast enzymes, respectively.

Maximum gap alignment of both the *T. brucei* and *L. donovani* AdoMetDCs with the reported eukaryotic and *E. coli* AdoMetDC sequences showed the parasite enzymes to be the most divergent of the eukaryotic proteins. Like the other eukaryotic proteins, however, the parasite AdoMetDCs shared essentially no homology with the *E. coli* enzyme. Despite the lack of overall homology to the other eukaryotic AdoMetDC primary structures, the *T. brucei* and *L. donovani* enzymes are similar to other eukaryotic AdoMetDCs in that they are synthesized as a proenzyme that is cleaved at a glutamyl-serine bond to produce the α- and β-subunits of the active enzymes. Thus, the cleavage sites of the proenzymes are identical to the cleavage sites of other eukaryotes, with high conservation of the surrounding eight amino acids. Southern blot analysis of the genomic DNAs revealed that the *T. brucei AdoMetDC* is present in multiple copies and is tandemly repeated at one locus, if not more, while the *L. donovani* gene is present as a single copy. Analysis of *T. brucei* and *L. donovani* mRNA by Northern blotting revealed that the coding sequence of the *AdoMetDC* genes

hybridized to single 1.8 kb and 3.1 kb transcripts, respectively.

Attempts to purify and characterize the AdoMetDC enzyme of *T. brucei* and *L. donovani* have previously been hindered by the inability to obtain large amounts of the protein. To obtain sufficient amounts of protein for enzymatic studies the coding regions of the respective genes were ligated into the bacterial expression vector pBAce, which utilizes the inducible bacterial *phoA* promoter to direct the expression of foreign genes in the multiple cloning site of the plasmid. The recombinant *T. brucei AdoMetDC* was expressed poorly; however, high levels of expression were obtained for the recombinant *L. dovovani AdoMetDC*, ranging from 40 - 60% of the total cellular protein. Although most of the recombinant *L. donovani* protein was present in the insoluble fraction in the form of inclusion bodies, a significant amount was soluble, corresponding to 50-100-fold greater levels of AdoMetDC activity (3 nmol of CO_2/h/mg of protein) than in promastigote *L. donovani* lysates and was sufficient to allow the biochemical characterization of the enzyme. The recombinant enzyme had a calculated K_m for S-adenosylmethionine of 76 μM.

To investigate whether the *L. donovani* AdoMetDC enzyme plays a regulatory role in the polyamine biosynthetic pathway, the pathway intermediate, putrescine, and product, spermidine, were tested for their effects on the activity of the AdoMetDC enzyme. The activity of the leishmanial AdoMetDC enzyme was stimulated approximately three fold over basal levels by the presence of 2 mM putrescine, with half-maximal stimulation at 50 μM. The putrescine stimulation of activity was strongly inhibited by micromolar concentrations of spermidine. Thus, it appears that the *L. donovani* AdoMetDC enzyme represents a classic regulatory enzyme, in a biochemical sense, being activated by an intermediate and

inhibited by the product of its pathway.

To obtain an additional tool with which to characterize the native AdoMetDC enzyme of both the wild-type *L. donovani* and MDL 73811 resistant mutant cells, rabbit antibodies were raised against the recombinant *L. donovani* AdoMetDC protein and were used to probe Western blots of SDS-PAGE separated promastigote parasite proteins. Western blots of parasites harvested during stationary phase showed two bands with the apparent molecular weights of the α- and β-subunits of the active enzyme, while blots of exponentially growing parasite proteins revealed a third band with an apparent molecular weight of the proenzyme.

In order to map the 5' end of the mature *AdoMetDC* transcripts from *T. brucei* and *L. donovani*, cDNA was synthesized and amplified using PCR and oligonucleotide primers corresponding to the spliced-leader sequence (SLS), which caps all mature mRNAs in these organisms, and an *AdoMetDC* specific sequence. Analysis of the amplified sequences indicated that the SLS attachment site on the leishmanial and trypanosomal *AdoMetDC* transcripts was 146 and 143 nucleotides 5' from the translational start sites, respectively.

To further characterize the chemotherapeutic mechanism of the adenosylmethionine analog, MDL 73811, a *L. donovani* derived cell line was generated by growing the parasites in sequentially increasing concentrations of the drug until a mutant was obtained capable of growing at concentrations which were 250 fold greater than lethal concentrations for wild-type cells. The *L. donovani AdoMetDC* gene was used to genetically analyze the possibility of gene amplification or transcriptional activation as a mode of drug resistance. Careful analysis of Northern and Southern blotting experiments indicated that both the copy number

of the *AdoMetDC* gene and the levels of its mRNA in mutants and wild-type cells were essentially equal. Likewise, biochemical studies showed no increase in AdoMetDC enzymatic activity in the mutant cell line as well and little difference in the sensitivities of the enzymes to inhibition by MDL 73811. From these results we conclude that it is unlikely that any modification of AdoMetDC activity or kinetic characteristics plays a role in the MDL 73811 drug resistant phenotype of the MDL 1000 mutant cell line.

As an outgrowth of our use of the *T. cruzi AdoMetDC* as an evolutionary stepping stone to isolate the *T. brucei AdoMetDC* gene, we wished to investigate the published hypothesis that *T. cruzi* is evolutionarily closer related to *T. brucei* than to *L. donovani*, e.g., that *T. cruzi* and *T. brucei* diverged from a common ancestor later than did *L. donovani*. Molecular phylogenic analysis of the protein sequences inferred here and of those reported for other AdoMetDC enzymes was generally in good agreement with published molecular phylogenies that used the highly conserved *small-subunit ribosomal RNA* (*rRNA*) gene sequences, predicting that the *Leishmania* and *Trypanosoma* genera are evolutionarily closely related and that they branched early from the eukaryotic lineage, as did other protozoan species. The phylogenic model constructed from the parasite AdoMetDCs, using the partial sequence obtained from the *T. cruzi AdoMetDC* PCR product, predicted however that *Leishmania* and *T. cruzi* diverged from a common ancestor later than *T. brucei* and are therefore the more closely related of the three organisms. This conflicts somewhat with the one previously published model. The methods employed to create the phylogeny and the results are discussed in greater detail in Appendix A.

INTRODUCTION

A. Parasitic Diseases

"And they journeyed from Mount Hor by way of the Red Sea, to compass the land of Edom...and the Lord sent fiery serpents among the people, and they bit the people: and much people died.... And the Lord said unto Moses, 'Make thee a fiery serpent and set it upon a pole; and it shall come to pass that everyone that is bitten, when he looketh upon it, shall live.'"

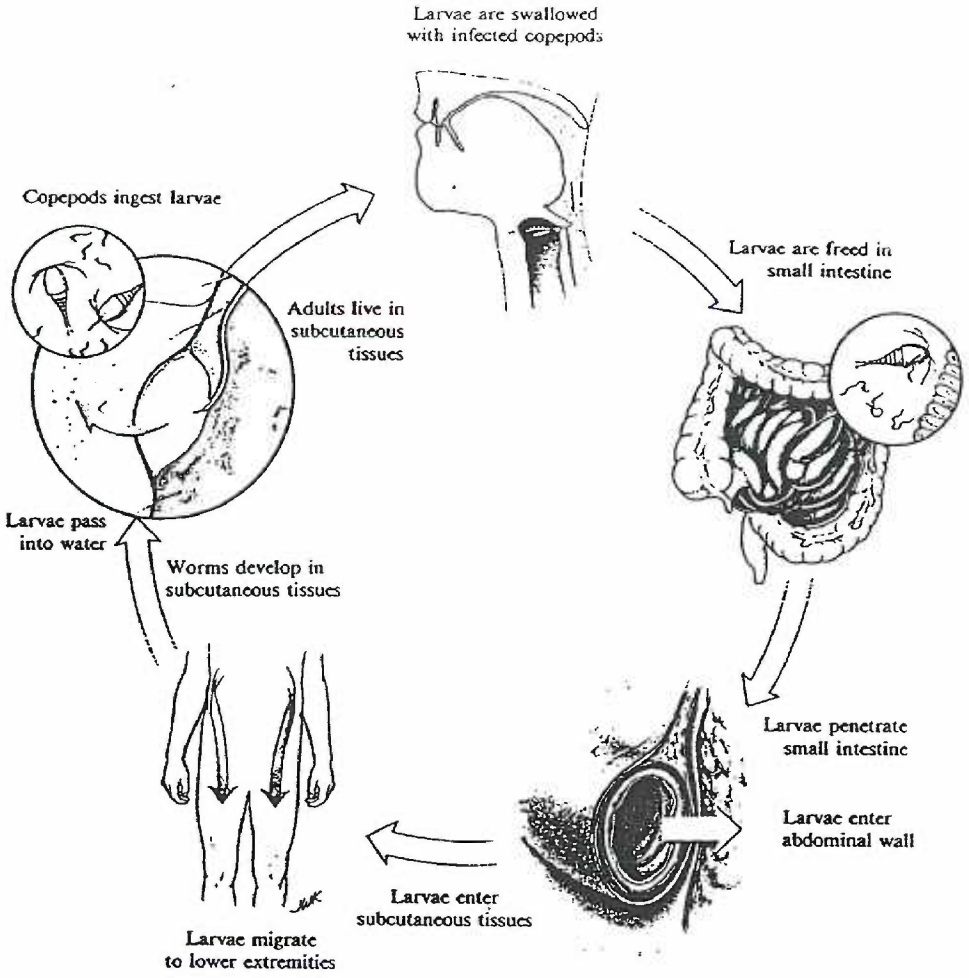
NUMBERS 21:6

The preceding passage is among the earliest and best read references to the intertwined existence of parasites and their human hosts. The quotation refers to the guinea fire worm, *Dracunculus medinensis*, whose larvae continue, even today, to contaminate the water supplies of the Middle East, Africa and Mediterranean basin, causing millions of infections and tens of thousand of deaths annually (Figure 1) (1). The meter-long adult female migrates to the skin, causing a burning ulcer, from which it must be extracted by rolling, centimeter by centimeter, around a small twig at a rate of one centimeter per day, over a period of about four weeks. This centuries old treatment, which is still practiced in many countries, is maintained symbolically in our society as the caduceus serpents, more commonly known as the emblem of the medical profession (Figure 2).

More recent works on infectious disease define a parasite as any organism that spends a portion of its life cycle in contact with and living at the expense of its host. Thus true parasites range in size from the 10-nanometer virions to the 10-meter long fish tape worm, *Diphyllobothrium latum* (2). The scientific discipline of parasitology has, however,

Figure 1. **Life cycle (above) and a 14th century wood cut (below) depicting the extraction of the guinea fire worm, *Dracunculus medinensis* (From "Medicine: A Treasury of Art and Literature. Macmillan Publishers, 1989).**

Dracunculus medinensis



Extraction of adult female worm from lesion.

Figure 2. **The two-headed serpent caduceus (above) and the symbol of American Medical Association and the medical profession (below).**


A·M·A
AMERICAN MEDICAL ASSOCIATION


M D

been further delineated into two major groups of eukaryotic organisms: the protozoa and the helminths, or worms. Annually, these two groups of organisms afflict nearly two thirds of the world's population and severely impact the public health and the economic development of developing countries throughout the world. The scope of human parasitic disease is easily illustrated by two species of blood sucking hookworms, *Necator americanus* and *Ancylostoma duodenale*, which infect nearly a billion people world-wide. Taking this number into consideration, these organisms consume a volume of blood on a daily basis roughly equivalent to that which would be obtained if every man, woman and child in New York city were to donate one unit of blood (1). More sobering, perhaps, is the fact that *Plasmodium falciparum*, the causative agent of malaria, continues to be the all time leading killer of all etiologies and its historical impact can be appreciated in light of the fact that half of all people who have lived and died have died of malaria (3).

Nearly equalling the breadth of the devastation caused by these organisms is their distinctive molecular biology and repertoire of unique biochemical pathways. It is this uniqueness that makes molecular and biochemical parasitology not only scientifically intriguing, but also of great utility in that many of the biochemical differences between the host and the parasite offer the opportunity for chemotherapeutic intervention.

It is against this backdrop of academic interest and practical necessity that this dissertation focuses on a rational chemotherapeutic target, S-adenosylmethionine decarboxylase (AdoMetDC), of two important protozoan parasites *Leishmania donovani* and *Trypanosoma brucei brucei.*

B. The Order Kinetoplastida

1. Classification

Leishmania and the trypanosomes belong to the Trypanosomatidae family and are known as kinetoplastid flagellates. They are a biologically diverse group of protozoans that are probably among the earliest organisms to diverge from our common eukaryotic ancestor (Figure 3) (4). Kinetoplastid flagellates, both free living and parasitic, are characterized by a single mitochondrion, the kinetoplast, which is unique in that it contains a network of thousands of topologically interlocked DNA circles. The main component of kinetoplast DNA (kDNA), comprising approximately 95% of the total kDNA, are the 0.7-2.5 kb minicircles (5,6). The remaining 5% of the kDNA is made up of approximately 50, 20 kb circular DNAs, that are analogous to the mitochondrial DNAs of most other eukaryotes (6). Specific kDNA sequences have recently been characterized and are currently utilized diagnostically to detect and classify human infections of *Leishmania* and trypanosomiasis (5,7,8).

The classification of each species and subspecies of *Leishmania* and the trypanosomes (Figure 4) is based on *in vitro* growth patterns of the promastigote as well as epidemiologic factors, isoenzyme analysis, and DNA-DNA hybridization studies (9,10).

Figure 3. **The evolutionary relationship of the trypanosomes and *Leishmania* to other common organisms as determined from *small-subunit rRNA* gene sequence similarities.** The relationship of the members of the Trypanosomatidae family to each other was inferred from the amino acid sequence similarities of the AdoMetDC enzyme as discussed in Appendix 1. The span between the nodes of the tree is proportional to the evolutionary distance between the organisms and is represented by the horizontal component of their separation in the figure (Adapted from reference 4). The relationship of *Trypanosoma cruzi* to *Leishmania* and *Trypanosoma brucei* as evolutionarily halfway between the two enabled us to use it as an evolutionary stepping stone. The deduced amino acid sequence from a *T. cruzi* AdoMetDC PCR product provided crucial sequence information, which allowed us to redesign our PCR primers so that they would amplify a segment of the *T. brucei* gene.

Evolutionary Relationship of the Trypanosomatidae Family to other Common Organisms

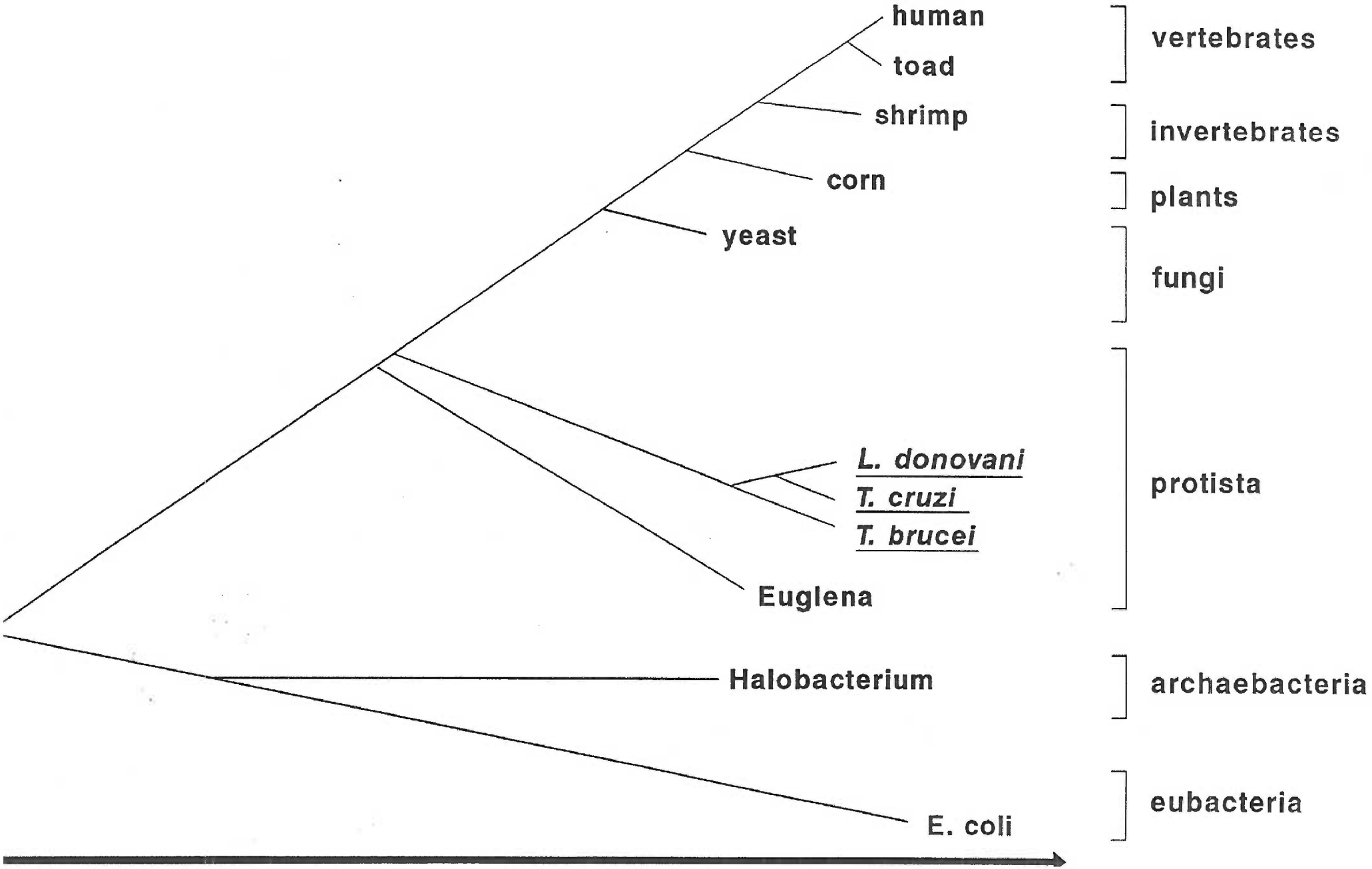
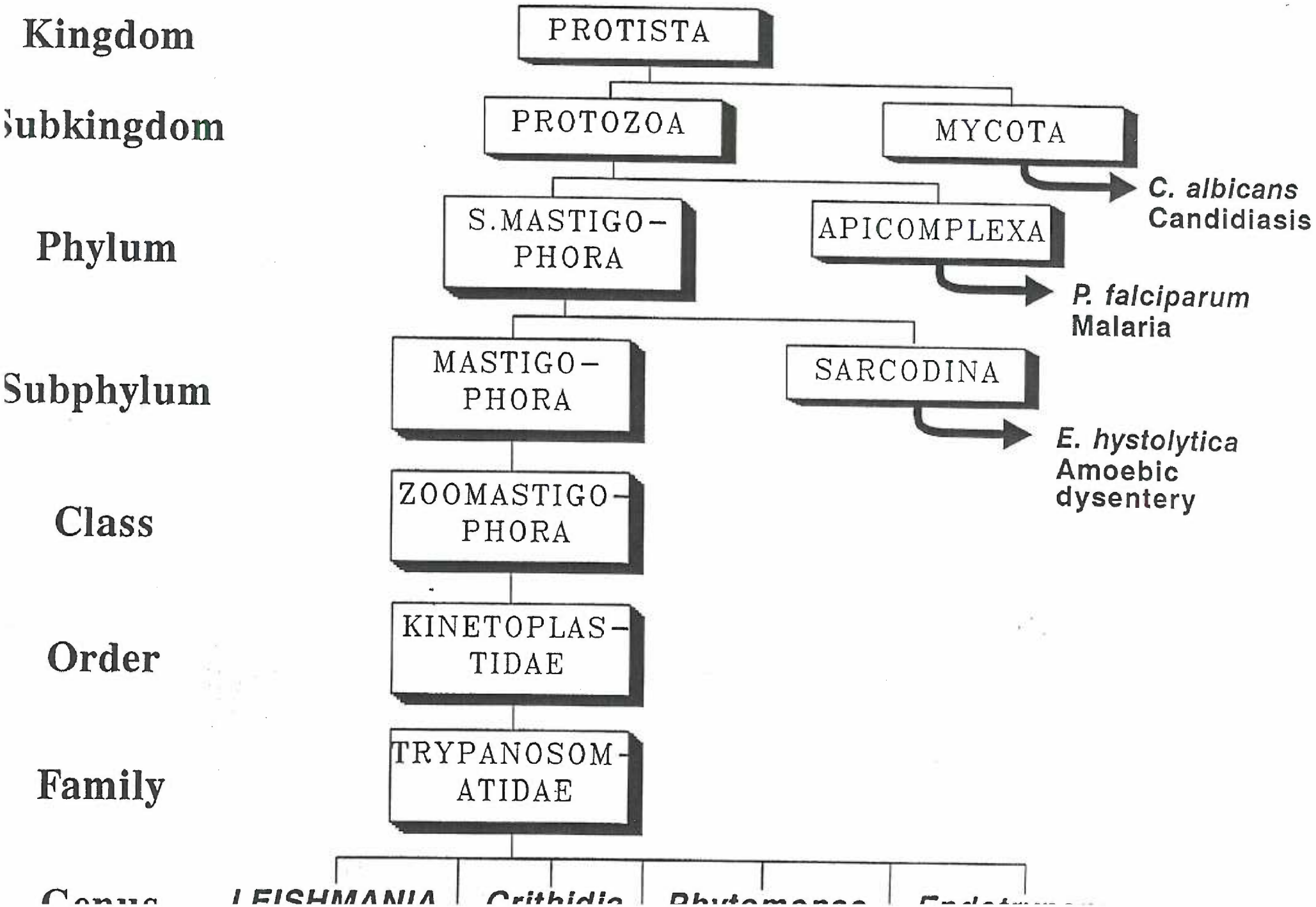


Figure 4. **Phylogenetic classification of the *Leishmania* and *Trypanosoma* genera, relative to other, common parasitic organisms from other phyla.**


Kingdom
PROTISTA
Subkingdom
PROTOZOA
MYCOTA
C. albicans
Candidiasis
Phylum
S.MASTIGO-PHORA
APICOMPLEXA
P. falciparum
Malaria
Subphylum
MASTIGO-PHORA
SARCODINA
E. hystolytica
Amoebic
dysentery
Class
ZOOMASTIGO-PHORA
Order
KINETOPLAS-TIDAE
Family
TRYPANOSOM-ATIDAE
Genus
LEISHMANIA
Crithidia
Phytomonas

2. *Leishmania*

a. History and Significance

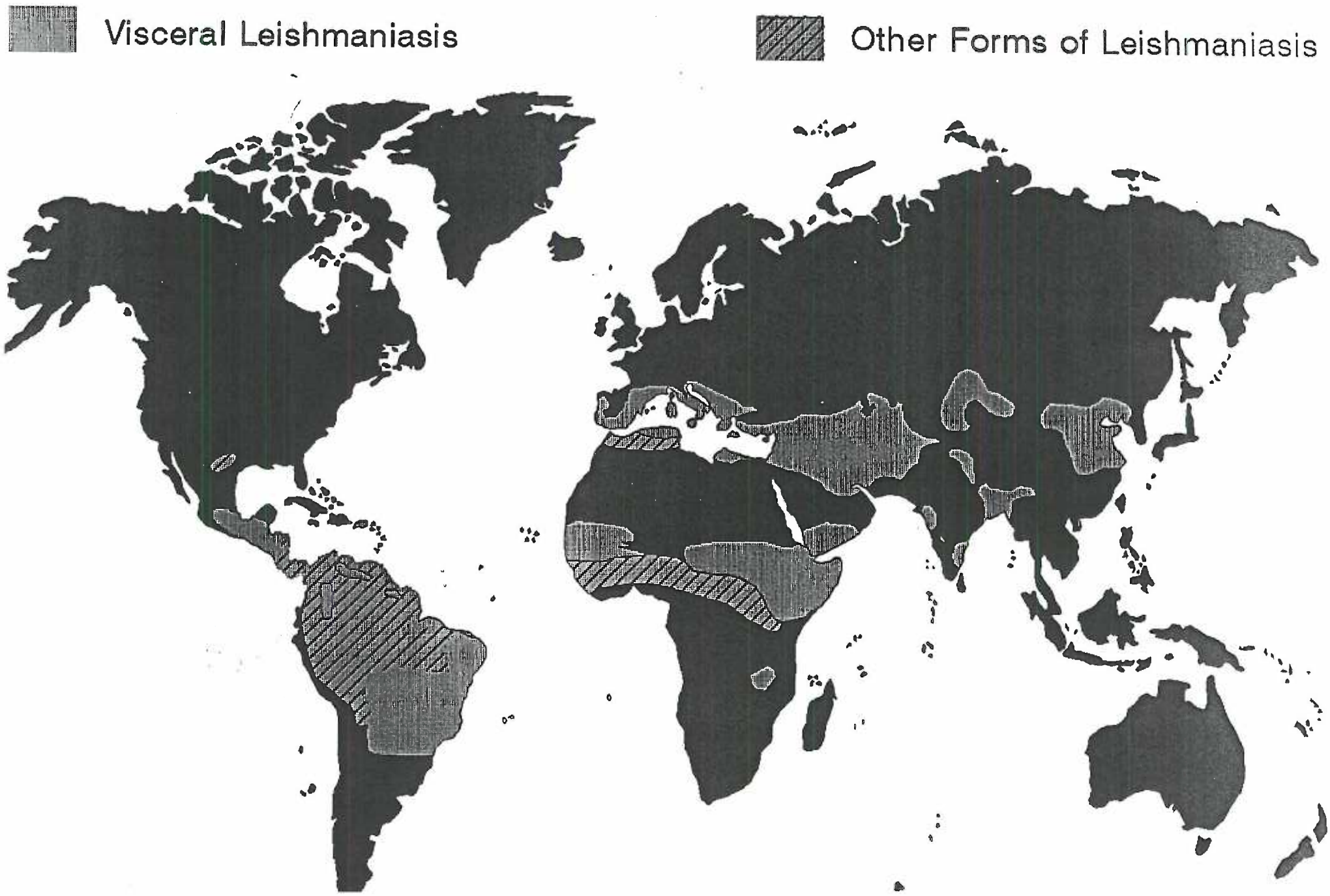
References to cutaneous leishmaniasis in the form of paintings on clay tablets and pottery have been found dating to the second millennium B.C. (11). However, it was not until 1903 that the agent of visceral leishmaniasis, *Leishmania donovani* (*L. donovani*) was independently identified by W.B. Leishman and C. Donovan working in India (1).

Leishmaniasis is endemic throughout the tropical and subtropical regions of the world (Figure 5). Annually the global incidence of new infections by the various species of *Leishmania* is between 400,000-600,000; however, hundreds of thousands more may be infected but go undetected due to the lack of adequate medical surveillance. Outbreaks of leishmaniasis impact the lives of millions (12) and continue to influence population movements in many regions of the world where the health care systems have been disrupted by war (13). As recently as 1991, a mass migration of the Sudanese Nuer tribe was linked to an epidemic of visceral leishmaniasis. Of the 2,714 refugees treated, 1,195 (44%) had clinical symptoms of visceral leishmaniasis (13).

Leishmania has also taken on a new significance as an opportunistic pathogen in transplant recipients, chemotherapy patients, and individuals with AIDS (14). Mediterranean countries such as Spain, France, and Italy have experienced dramatic increases in visceral leishmaniasis, most commonly associated with concomitant HIV infection (14-16). Further, the incidence of viscerotropic leishmaniasis, a form of visceral leishmaniasis caused by an *L. donovani*-related organism, *Leishmania major*, which is endemic to Saudi Arabia and the Arabic peninsula, has already been diagnosed in 35 Gulf

Figure 5. **Geographic distribution of leishmaniasis (adapted from information in reference 12).**

Geographic Distribution of Leishmaniasis



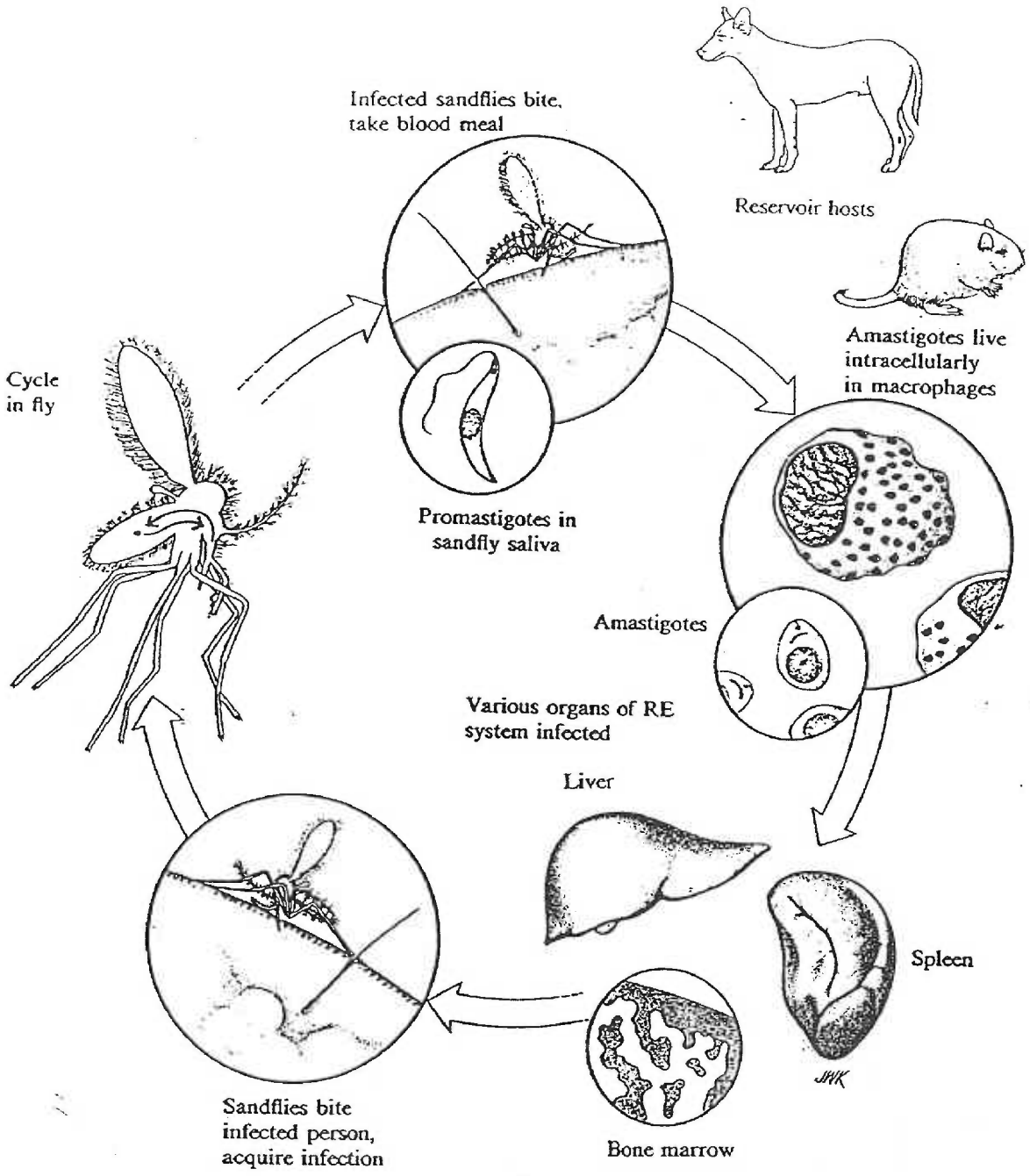
War veterans and it is likely that this number will continue to rise as more ill veterans are screened for the disease (17). Questions have also been raised about the safety of blood donations from all returning veterans. Because the incubation period of visceral leishmaniasis can be as long as two and a half years, blood donated from symptomless individuals can be a source of infection for an as yet undetermined length of time (18).

b. Life Cycle

In most endemic areas leishmaniasis is a zoonoses, or rather a disease of animals which is transmitted by an arthropod vector, in this case the female phlebotamine sandfly, to man as an incidental host (19). Only in India and certain African areas does man function as a reservoir for the infection of sandflies. Reservoirs for *Leishmania* vary from region to region; however, the best know examples include dogs (Asia and the Middle East), rodents (Saudi Arabia, East Africa, and regions of what used to be the Soviet Union), and opossums (Central and South America) (12).

Leishmania have two distinct morphologic stages in their life cycle: a flagellated, motile, insect-vector form called the promastigote and an aflagellate, nonmotile, intracellular form which infects the host macrophage, referred to as an amastigote (Figure 6). Infection begins when the host is bitten and inoculated by a female phlebotomine sandfly. The parasites are then phagocytized by macrophages and undergo a number of metabolic and morphologic changes characteristic of the amastigote stage, which is not only viable in, but literally thrives within the phagolysozome of the macrophage (19). Following the rupture of the infected macrophage the amastigotes remain either localized and repeat the cycle,

Life Cycle of *Leishmania donovani* and the Pathogenesis of Visceral Leishmaniasis



causing cutaneous leishmaniasis, or as is the case with visceral leishmaniasis, become disseminated and seed the organs of the reticuloendothelial system, e.g., the bone marrow, spleen, lymph nodes, and liver. The cycle is completed when another female sandfly takes a blood meal from an infected host and ingests macrophages containing parasites (1). Upon entering the environment of the sandfly stomach and subsequent release from the macrophage, the amastigotes undergo yet another morphologic and metabolic transformation, reverting to the original promastigote form (12).

c. Leishmaniasis as a Clinical Disease

The varied and distinctive lesions caused by *Leishmania* make leishmaniasis one of the more complex of the parasitic diseases. Traditionally, three major clinical diseases have been described, each caused by multiple species of *Leishmania*, with each species having its own distinct geographic distribution and epidemiologic characteristics (Table I.) (11,19). It has, however, been proposed that a fourth disease classification be created to include the viscerotropic leishmaniasis of the returning Gulf War veterans (17). The pathologic spectrum of leishmaniasis ranges from a self-limiting infection of the skin to the chronic and sometimes fatal systemic illness caused by *L. donovani* (20). The actual outcome of the infection, however, depends on the species and virulence of the infecting organism as well as the immune responsiveness of the host (19,21).

Both visceral leishmaniasis and viscerotropic leishmaniasis often present with flu-like symptoms, followed later by inflammation of the major organs of the reticuloendothelial system (11,20). Without treatment, the infection can, and often does, assume a chronic

Table I. **Clinical manifestations and geographic distribution of *Leishmania* species (compiled from references 1 and 19).**

Table I. Clinical manifestations and geographic distribution of syndromes of *Leishmania* species

Species	Clinical Manifestations	Geographic Distribution
Leishmania tropica major	"wet" cutaneous leishmaniasis	Middle East, Mediterranean basin
L. tropica minor	"dry" cutaneous leishmaniasis	Asia, Middle East, Europe, North Africa, India
L. aethiopica	disseminated cutaneous leishmaniasis	East Africa
L. mexicana mexicana	cutaneous leishmaniasis	Mexico, Central America, Amazon River Basin
L. m. amazonenzis	cutaneous leishmaniasis	Mexico, Central America, Amazon River Basin
L. m. pinfanoi	disseminated cutaneous leishmaniasis	Mexico, Central America, Amazon River Basin
L. braziliensis braziliensis	mucocutaneous leishmaniasis	Central and South America
L. b. guyanensis	mucocutaneous leishmaniasis	Guyana
L. b. panamensis	metastatic cutaneous leishmaniasis	Panama
L. peruviana	non-metastatic cutaneous leishmaniasis	Peru
L. donovani	visceral leishmaniasis	Asia, Southern Europe,

and fatal course, with the host generally succumbing to opportunistic infections (20). Among those that recover, either spontaneously or with treatment, 2-20% develop chronic cutaneous leishmaniasis characterized by hypersensitivity to leishmanial antigens similar to that seen in leprosy (1). Infected macrophages within the area of affected skin provide an important source of infection for sandflies (19).

Although cutaneous leishmaniasis invariably resolves without treatment, most forms of mucocutaneous and visceral leishmaniasis must be treated pharmacologically with intravenous pentavalent antimonials, usually sodium stibogluconate (Pentostam) or a related compound meglumine antimonate (Glucantime) over the course of several weeks to effect cures (11). Unfortunately because the pentavalent antimonials have been in use for over 70 years, resistance to these drugs has become both a clinical and epidemiologic problem (19). Resistant strains are usually treated with pentamidine isethionate or amphotericin B (22); however, both of these compounds have side effects which surpass even those of the antimonial drugs, ranging from liver disease and anemia to kidney failure, and their use is reserved for emergency situations (22).

Recent work on the chemotherapy of leishmaniasis has focused on the delivery of amphotericin B to the reticuloendothelial system in the form of liposomes, thereby greatly increasing treatment specificity and diminishing many of the adverse side effects (11). The technical support for this form of therapy unfortunately precludes its use in most of the countries with the highest incidence of leishmaniasis.

In summary, the clinical expression and treatment outcome of human leishmaniasis depends not only on the virulence of the infecting species, but also on host factors such as

nutritional and immunologic status (21). The design of more effective therapeutic regimens for the treatment of leishmaniasis will not only have to circumvent the problem of drug resistance, but be customized to the immune status of the patient as well.

d. Prevention and Control of Leishmaniasis

The control of leishmaniasis in most regions is confounded by the fact that humans are only an incidental host to a large reservoir of parasites which are maintained by cycles of infection in wild animal populations (19). Due to the epidemiologic problems this poses, immunization is the most attractive alternative. Unfortunately, an effective vaccine has yet to be developed despite an intensive research effort and until such measures are developed, literally millions of lives are dependent on the development of suitable alternative measures to combat leishmaniasis (23,24).

A variety of traditional methods have been employed to prevent the spread of leishmaniasis involving vector control, forms of immunization, and elimination of the animal reservoir populations. One of the most successful control methods from an epidemiologic viewpoint involved the spraying of dichloro-diphenyl-trichloroethane (DDT) to control the anopheles mosquito population and malaria (1). A byproduct of this effort was the eradication of phlebotamine sandfly vector of *Leishmania,* and leishmaniasis, in the overlapping areas. Concern about the ecologic consequences of this program and the development of DDT resistant mosquitos led to the termination of this practice and with it the control of the sandfly population and a resurgence in leishmaniasis. The more limited application of insecticides around dwellings also has an important effect, because the female

sandfly typically rests on window sills and door frames before and after taking her blood meal (159).

Although no immunization exists for the more serious forms of leishmaniasis, a form of vaccination for cutaneous leishmaniasis has been practiced for centuries in the Middle East (1). To avoid unsightly scarring of the face, hands and arms, adults inoculated their children with viable organisms on the back of the leg, where such a scar would not be perceived as disfiguring (12). The resulting immunity prevented further infection and disfigurement and eased the selection of an arranged marriage!

3. *Trypanosoma brucei*

a. History and Significance

"The long history of West African civilization is...the history of the evolution of human societies along paths that have enabled them to avoid frequent periodical decimation by trypanosomiasis."

John Ford 1971 (25)

African trypanosomiasis, as a human disease, is as ancient as humanity itself. The first references to African trypanosomiasis appear in written records in the 14th century; however, it was not until the European colonization of subsaharan Africa, with the construction of roads and the development of river travel, that epidemics occurred (6,26). Particularly devastating epidemics in Uganda and Kenya in the early 1900s took the lives of nearly two thirds of the population (27). More recent outbreaks in the late 1980's in Uganda, Kenya, and Angola also caused considerable mortality (26-28). It is the memory of these events and the constant threat of recurrent epidemics that forces the developing countries of this region to spend a disproportionate amount of public health dollars on trypanosome control measures despite a relatively low average annual incidence of 25,000 to 50,000 infections (26,29,30).

Adding to the economic burden is the fact that trypanosomes parasitize not only humans but wild animals and livestock as well. *Trypanosoma brucei brucei*, besides being the model organism for scientific studies such as this dissertation, causes a chronic wasting disease of cattle and domestic animals, making it of major economic importance by preventing the farming of a portion of equatorial Africa roughly equivalent to the size of

the United States (1).

b. Life Cycle

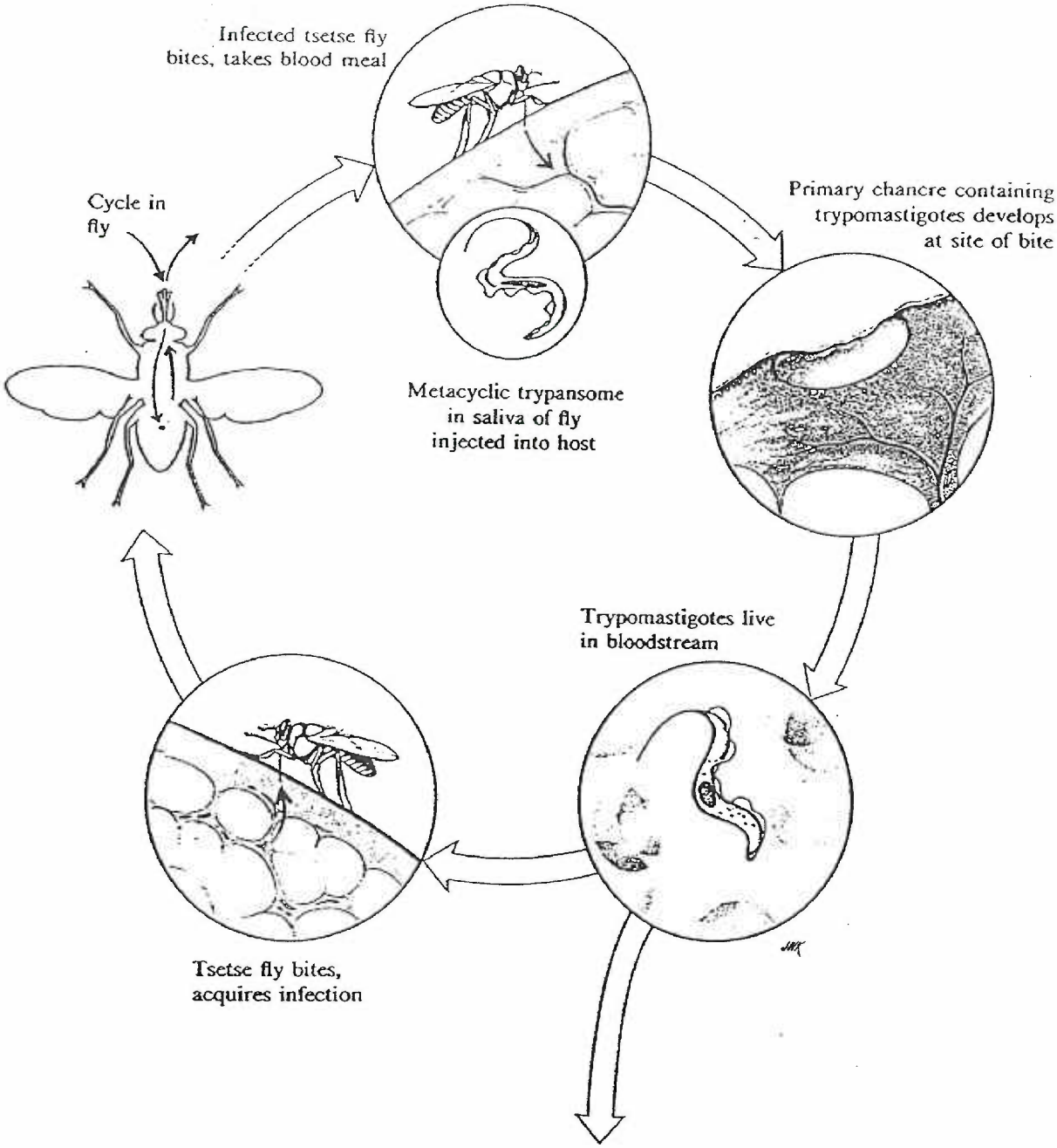
Like leishmaniasis, trypanosomiasis is both a zoonosis and a human disease. The more virulent East African trypanosomiasis is predominately a disease of wild game such as the hartebeest, humans being an incidental host. The less virulent West African trypanosomiasis is generally classified as a human disease and does not have a significant animal reservoir. Both the East and West African trypanosomes are transmitted by the bite of both sexes of various species of tsetse fly of the genus Glossina. During a blood meal, infective metatrypanosomes are injected intradermally and undergo a rapid morphologic and metabolic transformation to a blood-stream form, or trypomastigote (Figure 7). Trypomastigotes are transported via the local lymphatics to the circulatory system and the cycle is completed when another fly takes a blood meal (12). Tsetse flies are strong fliers and remain infected for the duration of their two to three month life span, delivering an estimated 40,000 trypanosomes into their host with each blood meal (minimum infective dose = 300-500)(1). The tsetse fly-trypanosome duo has thus evolved into a devastatingly efficient disease "team", that can transmit sleeping sickness over a large area for relatively long periods of time.

c. African Trypanosomiasis as a Clinical Disease

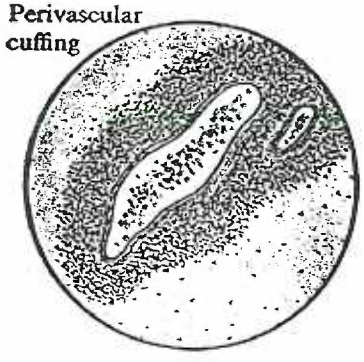
Like leishmaniasis, the immune responsiveness of the host plays an important role in the clinical picture and treatment outcome of African trypanosomiasis (21). However,

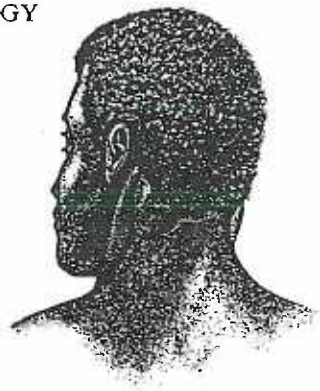
Figure 7. **Life cycle of *T. brucei rhodesiense* and the pathogenesis of African trypanosomiasis (adapted from reference 1).**

Life Cycle of *Trypanosoma brucei* and the Pathogenesis of African Trypanosomiasis



PATHOLOGY





Winterbottom's sign

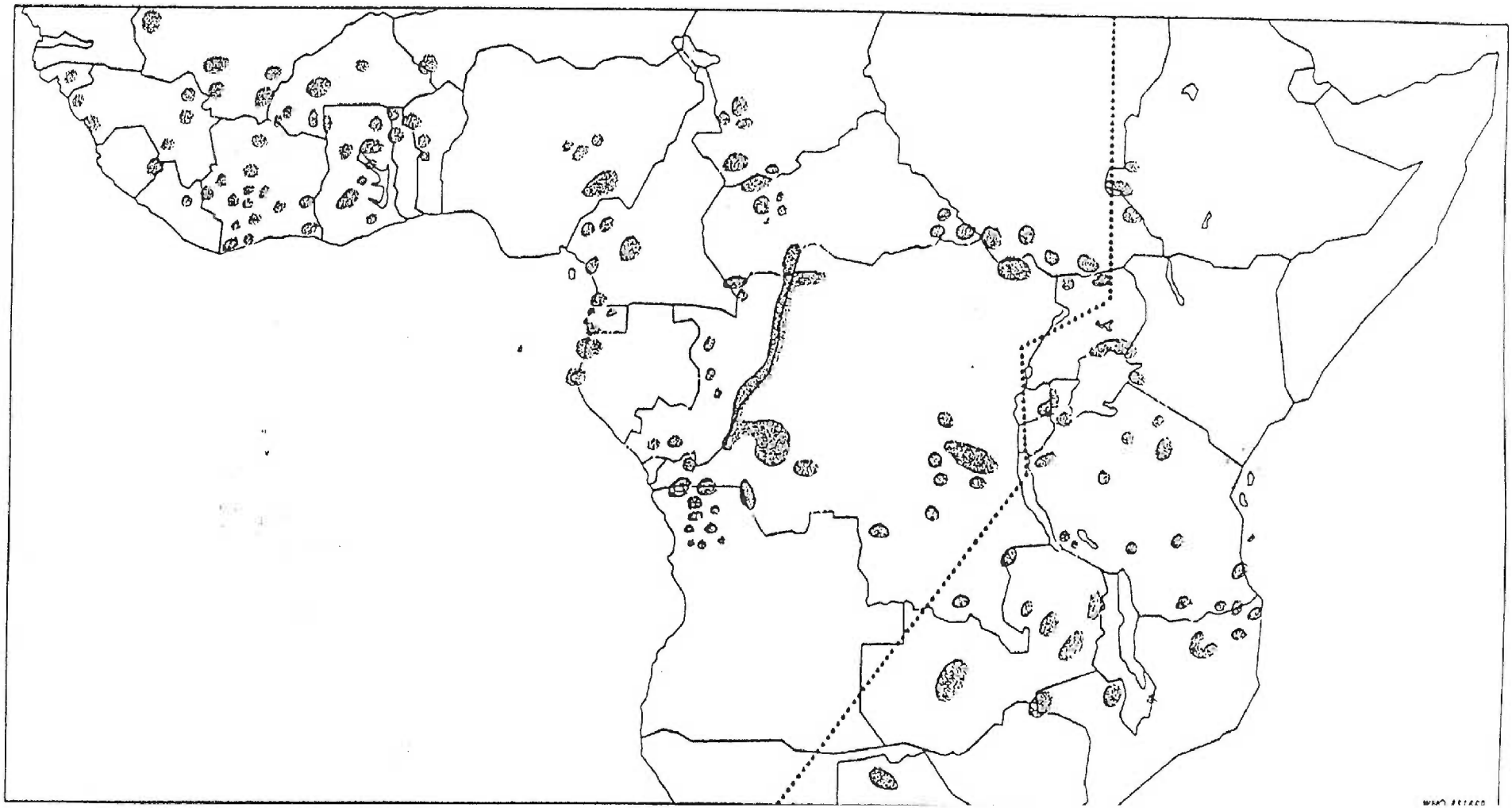
unlike leishmaniasis, African trypanosomiasis is almost always fatal if untreated, sometimes within weeks (12). Trypanosomes are infamous for the grace with which they evade the host's immune system. Their unique form of antigenic variation is based on the periodic rearrangement of membrane glycoproteins. Thus, just as antibodies appear that are capable of clearing one parasite population, a new, variant antigenic subpopulation arises, in a game of cat and mouse to which the host eventually succumbs (28).

Human African trypanosomiasis is caused by two subspecies of *Trypanosoma brucei*: *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *T. brucei rhodesiense* (*T. b. rhodesiense*). Although both forms of sleeping sickness are fatal if untreated, they differ in the onset and duration, as well as their geographic distribution (Figure 8) (28,31). *T. b. gambiense* is predominant in West Africa and causes a chronic form of sleeping sickness with an insidious onset and a variable course that can last from several months to several years (28). Presenting with flu-like symptoms, the West African disease also causes a number of non-specific central nervous system abnormalities ranging from Parkinsonian states to outright mania or depression (20). Interestingly, it is not uncommon for patients who have been committed to psychiatric hospitals in this region to be subsequently diagnosed with West African sleeping sickness (20,31).

By contrast, *T. b. rhodesiense* predominates in East Africa and is the agent of a fulminant disease lasting only a few weeks or months (20,28). East African sleeping sickness invariably presents with malaise and lymphadenopathy. The swelling of the lymph nodes at presentation is particularly evident in the posterior cervical area and is so characteristic that it is said to be virtually diagnostic of trypanosomiasis (1,32).

Figure 8. **Geographic distribution of the East African (*Trypanosoma brucei rhodesiense*) and West African (*Trypanosoma brucei gambiense*) forms of trypanosomiasis (adapted from reference 28).**

DISTRIBUTION OF SLEEPING SICKNESS FOCI IN AFRICA: *T. brucei gambiense*, areas of distribution in West and Central Africa correspond to those left of the dotted line; *T. brucei rhodesiense*, areas of distribution in East Africa correspond to those right of the dotted line.



The progression of human trypanosomiasis is generally divided into two stages. Stage one involves systemic infection before trypanosomes have invaded the central nervous system (CNS) and stage two involves systemic infection with CNS involvement (20). This staging system has practical significance because the most effective drug for treating both subspecies is Suramin, which does not cross the blood-brain barrier (22). After invasion of the CNS by *T. b. gambiense*, α-difuoromethylornithine (DFMO) is still effective. However infections involving *T. b. rhodesiense*, which is mysteriously resistant to DFMO, force physicians to employ the arsenical drug Melarsoprol, which has a number of very severe side effects (22,33). Both DFMO and Melarsoprol are discussed elsewhere in greater detail.

d. Prevention and Control of African Trypanosomiasis

The fact that our ancestor, *Homo erectis*, evolved in the same region that is home to the African trypanosome, gives one a healthy perspective as to the impact and importance of this disease, past, present, and future, on the human species. Despite the fact that man has been struggling with the epidemiologic patterns of the African trypanosome for millions of years, it was not until 1930 that successful attempts were made to control *T. b. gambiense* in West Africa by systematically clearing brush and trees from large stretches of river banks and paying a "bounty" for trapped flies (26). Later, with the advent of effective insecticides, such as DDT and dieldrin, the spraying of the riverine habitat of the *T. b. gambiense* vector became a viable alternative which continues to be successfully implemented today when not disrupted by war, famine, or politics (34).

Control of *T. b. rhodesiense* in East Africa is more complex due to the wide

distribution of the vector species over its bush and savanna habitat (26). The large area involved and the corresponding volume of chemicals needed to spray such a region raises serious concerns as to the ecological impact of widespread insecticide use. Spraying for Rhodesian sleeping sickness control has been reserved for epidemic situations, as occurred in the early 1980's (34).

Unlike the situation with *Leishmania*, no traditional form of immunization has ever been practiced for trypanosomiasis and the prospects for the development of a viable commercial vaccine appear far less promising than for *Leishmania* (24).

In conclusion, it appears that vector control, although effective for the prevention of Gambian sleeping sickness is still problematic for Rhodesian sleeping sickness except in epidemic situations. Thus, the discovery of more specific and efficacious trypanocides remains a necessity if this region of Africa is to remain inhabited and realize its productive potential.

C. Rational Drug Design

1. The Concept of Directed Drug Design

The available therapeutic regimens for human trypanosomiasis, leishmaniasis and many other tropical and subtropical diseases trail far behind the incredible advances made in treatment of bacterial diseases over the past 40 years. Furthermore, many of the drugs used in the therapy of trypanosomiasis and leishmaniasis were first developed over 50 years ago and would not pass the more stringent standards of drug safety observed today (8,35). Drug discovery and development has traditionally been a complex, costly, and largely empirical process. Because parasitic diseases occur in poor underdeveloped countries, there has been little incentive for pharmaceutical companies to invest the millions necessary to develop new drugs through empirical methods (36,37).

Rational drug design seeks to circumvent the costly empiric methods by using the knowledge obtained from both biochemical and structural studies to expedite biochemical and pharmacologic research. Although in its infancy and only theoretical speculation in the late 1970's, rational drug design is now coming of age and is being effectively used to design therapeutic inhibitors of sialidases of the influenza virus (38,39). Inhibitor design begins with the isolation and biochemical characterization of the protein target and necessitates a fundamental understanding of the pathogen's biochemistry (35). In the development of chemotherapeutic agents for infectious diseases these targets can be broadly categorized into three areas: 1) unique pathogen enzymes, 2) structural proteins that are indispensable for the parasite and circumventable by the host, and shared enzymatic pathways that differ in either their regulation or their biochemical and pharmacologic characteristics (40).

Once the protein target has been identified, both biochemical and biophysical studies can be employed to develop computer-generated models of the active site or ligand binding motifs. Nuclear magnetic resonance (NMR) and X-ray crystallography are presently the only two biophysical methodologies available to obtain the high resolution necessary for structure based design (41). Both techniques require large amounts of very pure protein. Although not trivial, this is now possible through the use of modern recombinant DNA technology. NMR technology is currently effective for proteins less than 15 kDa in size. For larger proteins one must rely on X-ray crystallographic methods, the primary obstacle of which is the time and care, sometimes years, needed to prepare protein crystals capable of diffracting to the high resolution which is necessary (42). In the future, as more protein structures are solved, it may ultimately be possible to obtain a substantial amount of three-dimensional information from primary sequence alone (41). Along these lines, the Tropical Disease Research Program of the World Health Organization currently encourages basic scientific research on a multitude of biochemical pathways or unique structures in the parasitic protozoa which fit these criteria (43).

S-adenosylmethionine decarboxylase (AdoMetDC) is a target for structure-based drug design and is a key regulatory enzyme in the polyamine biosynthetic pathway as well as being integrally involved in three other promising kinetoplastid pathways: purine metabolism, trypanothione synthesis, and methionine recycling. For these reasons AdoMetDC and other enzymes of the polyamine biosynthetic pathway are regarded as promising rational chemotherapeutic targets (44,45).

2. Polyamines

a. Structure

The polyamines putrescine, spermidine and spermine comprise a class of naturally occurring compounds that possess two, three, and four positive charges at physiologic pH, respectively (Figure 9) (46). Chemically, spermidine and spermine are organic aliphatic polycations created by the repetitive addition of an aminopropyl moiety to putrescine (46). In contrast to the higher eukaryotes which contain both spermidine and spermine, the former is the principle polyamine found in the trypanosomes and *Leishmania* (47).

b. Physiologic Importance

The polyamines and their derivatives are ubiquitous in nature and are essential for normal growth and differentiation in all eukaryotes and most prokaryotes (48-50). The actual cellular concentrations of the polyamines varies greatly: from 30 mM in actively dividing *Esherichia coli* (*E. coli*) to less than micromolar levels in quiescent eukaryotic cells (43). Interestingly, several prokaryotes exhibit growth in the absence of polyamines (e.g., *E. coli*), but at markedly reduced rates (49).

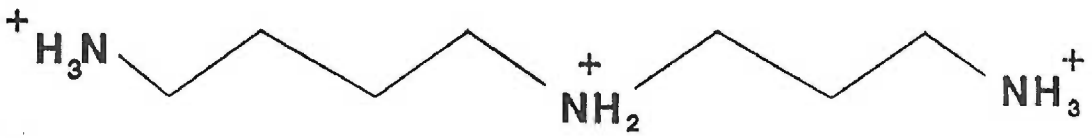
Polyamines exert a plethora of biological effects. As polycations, they stabilize the conformation of negatively-charged molecules and structures such as nucleic acids, membrane-bound organelles, and the transcriptional and translational machinery of the cell (50,51). For example, a derivative of spermidine, hypusine, is covalently bound to the translationally-active form of the eukaryotic initiation factor, eIF-4D (50).

Beyond these generalizations, however, little is known about the specific *in vivo*

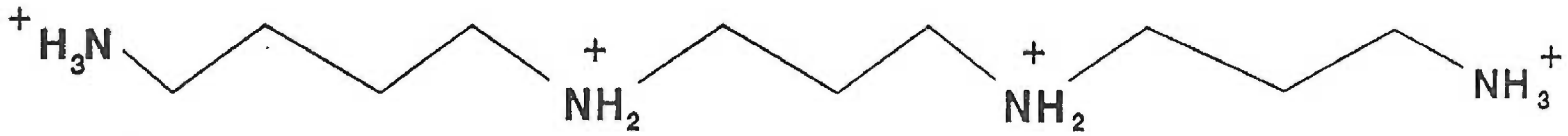
Figure 9. **Structures of the physiologically significant polyamines** (Note: For the sake of brevity putrescine is referred to as a polyamine, despite the fact that it is actually a diamine).

PHYSIOLOGICALLY SIGNIFICANT POLYAMINES

Putrescine



Spermidine



Spermine

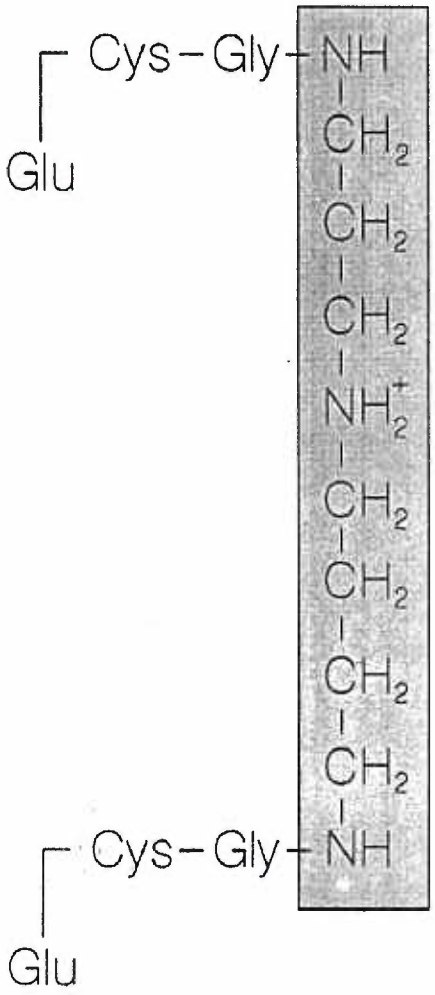
molecular functions of the polyamines in eukaryotes. Interestingly, spermidine has an added significance in *Leishmania* and the trypanosomes. These parasites contain a unique oxidant defense system in which glutathione is replaced by a spermidine containing glutathionyl-spermidine molecule, commonly referred to as trypanothione. Accordingly, glutathione reductase is replaced by the parasite enzyme, trypanothione reductase (Figure 10) (52). These differences have obvious therapeutic relevance since the synthesis of trypanothione can be blocked by inhibition of either ornithine decarboxylase (ODC) or AdoMetDC, thereby subverting the parasite antioxidant defenses, while leaving those of the host intact (33,35,36).

c. The Biosynthetic Pathway

In the kinetoplastid flagellates the synthesis of spermidine is accomplished via the concerted action of three enzymes: ODC, AdoMetDC, and spermidine synthase (Figure 11.). In higher eukaryotes and mammals a fourth enzyme, spermine synthase, is necessary for the addition of another aminopropyl group from decarboxylated S-adenosylmethionine to spermidine to produce spermine; however, this enzyme is not present in *Leishmania* and the trypanosomes. A key difference in the polyamine metabolism of *Leishmania* and the trypanosomes is that *Leishmania* may have an efficient transport system for polyamines and is likely able to circumvent the therapeutic inhibition of the polyamine pathway by inhibitors such as difluoromethylornithine (described in greater detail later) by uptake of host polyamines.

Figure 10. **Comparison of the structures of glutathione and trypanothione** (the spermidine molecule is boxed).

Trypanothione



Glutathione

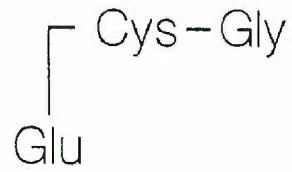
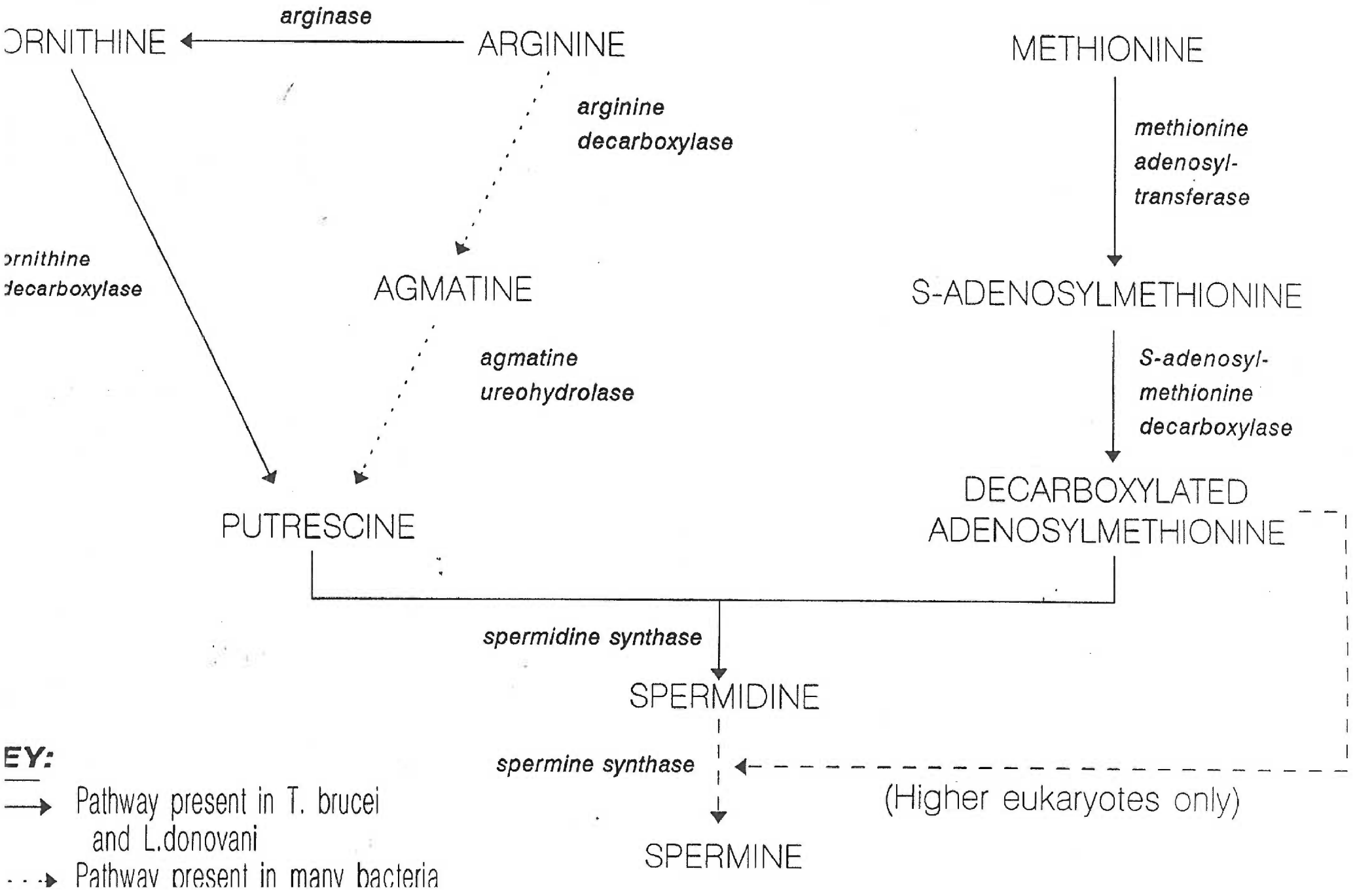


Figure 11. **The polyamine biosynthetic pathway.**

THE POLYAMINE BIOSYNTHETIC PATHWAY



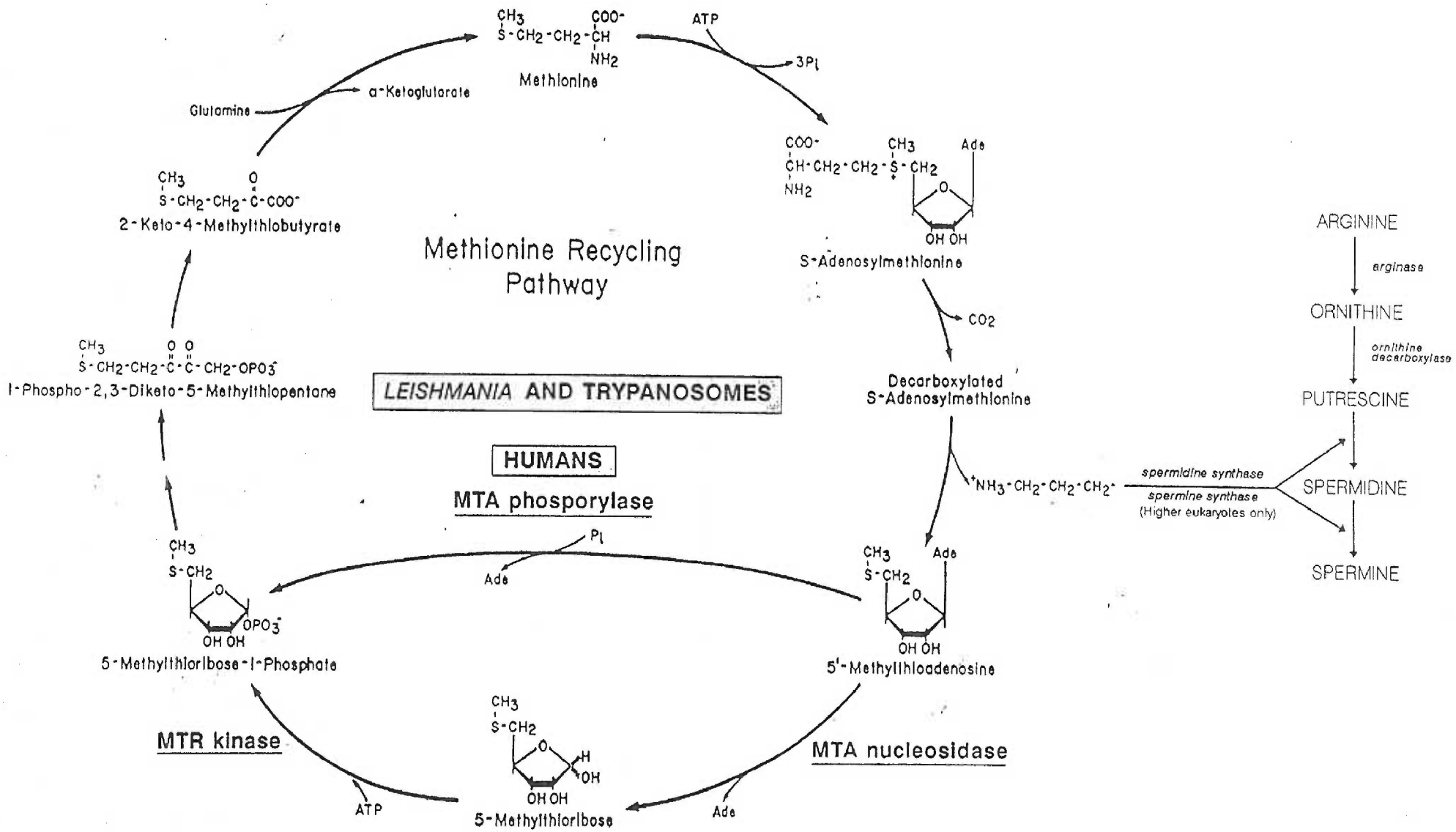
d. Methionine Recycling

Although still being characterized, further host-parasite differences appear to exist in the metabolism of decarboxylated S-adenosylmethionine after the loss of its aminopropyl group. Methylthioadenosine (MTA), the methionine containing adduct of the spermidine synthase reaction, is toxic if allowed to accumulate, and is obligatorily recycled via methylthioadenosine phosphorylase (MTA phosphorylase) in humans and the kinetoplastid parasites (Note: Many other parasitic protozoa utilize an alternative pathway via methylthioadenosine nucleosidase and methylthioribose kinase) (Figure 12) (53). The parasite MTA-phosphorylase is purported to have a much broader substrate specificity than the human enzyme and it has been proposed that MTA analogs could be used to activate toxic adenine analogs and possibly inhibitors of AdoMetDC as well (53). This has direct implications for the rational design of therapies for leishmaniasis, because all drugs which target *Leishmania* must first enter the macrophage either by diffusion or active transport, thereby necessitating that the compound be innocuous to the host cell.

e. Regulation of Polyamine Synthesis

Polyamine synthesis in mammalian cells is under stringent control. AdoMetDC seems to be a key regulatory point of the pathway, the human form of which has now been extensively characterized (54). First, the transcription of mammalian ODC and AdoMetDC are induced by trophic stimuli such as injury in liver cells or estrogen exposure in vaginal epithelium (55). AdoMetDC activity is further regulated through putrescine stimulation of mRNA translation, enhancement of proenzyme processing to the active α and

Figure 12. **The methionine recycling pathway.**


Methionine Recycling Pathway
Methionine
ATP
3Pi
S-Adenosylmethionine
CO2
Decarboxylated S-Adenosylmethionine
LEISHMANIA AND TRYPANOSOMES
HUMANS
MTA phosporylase
Pi
Ade
5'-Methylthioadenosine
MTA nucleosidase
5-Methylthioribose
ATP
MTR kinase
5-Methylthioribose-1-Phosphate
1-Phospho-2,3-Diketo-5-Methylthiopentane
2-Keto-4-Methylthiobutyrate
Glutamine
α-Ketoglutarate
spermidine synthase
spermine synthase
(Higher eukaryotes only)
ARGININE
arginase
ORNITHINE
ornithine decarboxylase
PUTRESCINE
SPERMIDINE
SPERMINE

β subunits, and allosteric activation of enzymatic activity (see Section 4) (55). Finally the end product of the polyamine pathway, spermine, inhibits putrescine activation (56). Prior to the isolation of the genes encoding AdoMetDC from *L. donovani* and *T. b. brucei*, little was known about regulation of the polyamine pathway in kinetoplastids. From the conserved amino acid residues and kinetic studies of the parasite AdoMetDCs, it is now known that these organisms are also fastidious regulators of their polyamines via AdoMetDC. This tight regulation of AdoMetDC appears necessary due to the high metabolic cost of producing S-adenosylmethionine and also because no other known biochemical reactions use decarboxylated S-adenosylmethionine (54).

It also appears that the enzymatic half-lives of ODC and AdoMetDC are regulated by polyamine levels, although the details of this regulation and how it relates to the PEST hypothesis (Section E) have yet to be fully elucidated (45).

3. Alpha-Difluoromethylornithine in Chemotherapy

Prior to the approval of α-difluoromethyornithine (DFMO) for Gambiense sleeping sickness in 1990, the only available drug for the treatment of late stage African trypanosomiasis with central nervous system involvement was melarsoprol (57). As an arsenical which must be dissolved in propylene glycol, melarsoprol exhibits a number of dangerous side effects. Perhaps the most notorious aspect of the drug being a reactive encephalopathy in up to 10% of the patients receiving treatment within the first 10 minutes with fatalities ranging from 5 to 10% (Note: And that's just from the treatment) (22,33). In stark contrast, chemotherapy with DFMO is rarely associated with any severe or

permanent side effects and is now the most effective drug available for the treatment of advanced, CNS stage, West African trypanosomiasis (20,57).

Mechanistically, DFMO is an ornithine analogue that acts as an enzyme-activated suicide inhibitor of ornithine decarboxylase (ODC) (Figure 13) (47). A number of mechanisms have been proposed to explain the parasite-specific trypanocidal action of DFMO. Foremost is the preferential inactivation of the parasite ODC and depletion of cellular polyamines. The parasite ODC enzymatic half-life is reported to be greater than 6 hours while that of the human is approximately 10 minutes. Thus, inactivated parasite enzyme cannot be immediately replaced whereas the host enzyme is readily resynthesized to provide polyamines for host cellular metabolism (58). This explanation is not without controversy. Others have suggested that a paradoxical inactivation of AdoMetDC occurs as a result of decreased putrescine levels, resulting in a concomitant rise in adenosylmethionine leading to toxicity in the parasite via aberrant methylation (59) as well as through depletion of spermidine for trypanothione synthesis which is required for the parasite's oxidant defenses.

Although DFMO has revolutionized the treatment of West African trypanosomiasis and has been called a "resurrection drug" by some, at $500 per cure (equivalent to about $20,000 per cure in the U.S.) it is far from cost effective and must be administered in large quantities, i.e., one gram per dose, four times daily, for 14 days (28). An additional drawback is that DFMO is rarely effective for the more fulminant East African trypanosomiasis. Ideally, a trypanocide must be developed that is not only safe and affordable, but which exhibits far better pharmacokinetic characteristics than DFMO.

Figure 13. **Comparison of the natural substrates for ODC and AdoMetDC with their respective suicide-substrates: DFMO and MDL 73811.**

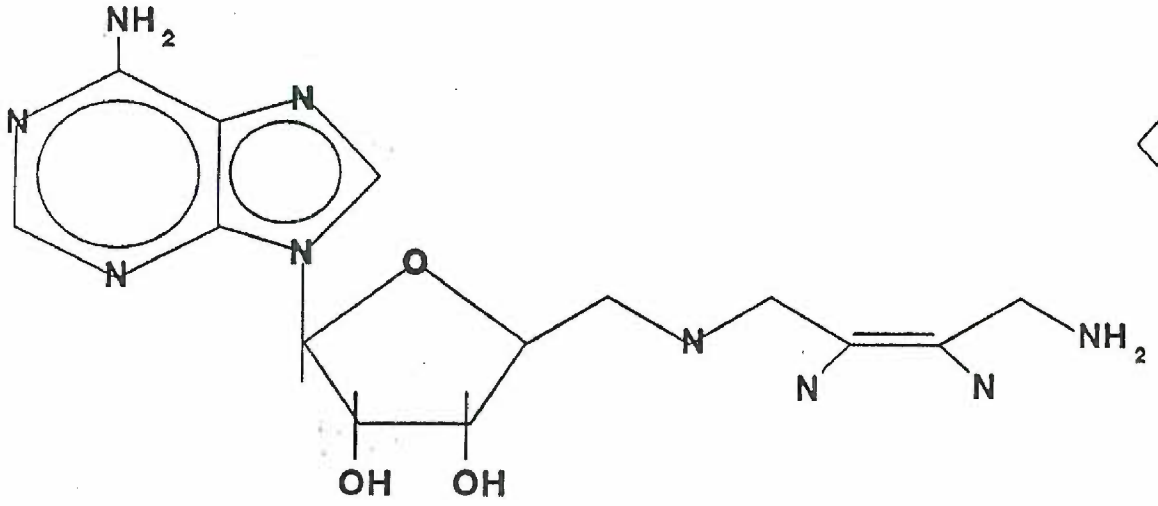
SUBSTRATE ANALOGS OF THE POLYAMINE PATHWAY

Analogs

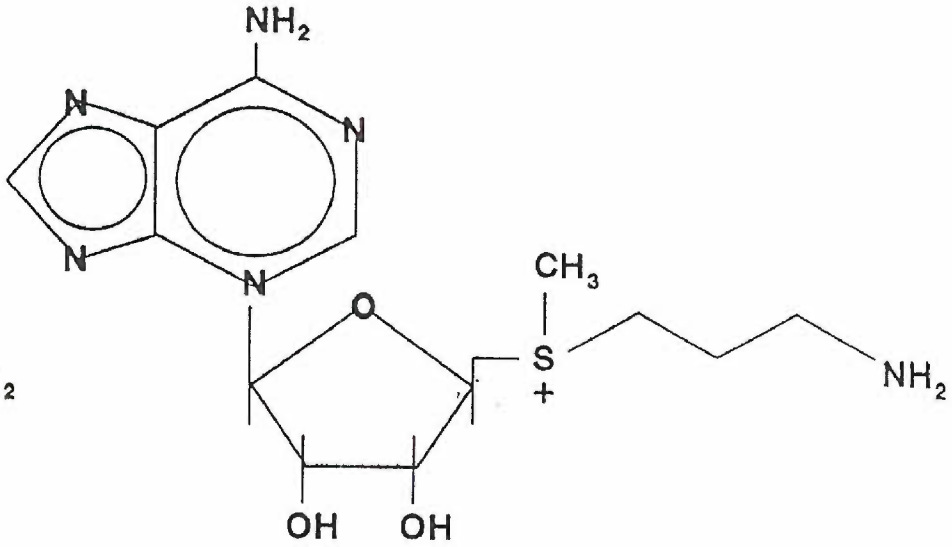
Natural Substrates

5'-{[(Z)-4-Amino-2-butenyl]methylamina}-
5'-deoxyadenosine

MDL 73811



S-Adenosyl-L-Methionine



α -Difluoromethylornithine

DFMO

Ornithine

DFMO is also toxic to cultured leishmanial promastigotes (60); however, it appears to be much less effective in the treatment of leishmaniasis. This is likely due to the fact that *Leishmania*, unlike trypanosomes, which do not possess an efficient polyamine transporter, have the ability to at least partially utilize host polyamines and can thereby circumvent the parasite-specific inhibition of polyamine biosynthesis (61). Therapeutic inhibitors of polyamine metabolism in *Leishmania* may therefore require the development of a coinhibitor of the parasite's polyamine transporter to be effective.

4. S-Adenosylmethionine Decarboxylase as a Chemotherapeutic Target

For over 20 years, AdoMetDC has been considered a target for the chemotherapy of proliferative diseases, e.g., malignancies, on the basis of the observation that the cytotoxic antiproliferative drug, methylglyoxal bis(guanylhydrazone) (MGBG), is also a potent inhibitor of the AdoMetDC enzyme (45). Although its severe side effects have precluded its use in the treatment of human African trypanosomiasis, MGBG cures murine models of the infection (62). Along these same lines, Berenil and pentamidine, two anti-trypanosomal drugs, also inhibit AdoMetDC as just one of their many effects on trypanosomes (62). More recently, a trypanocidal inhibitor of AdoMetDC, MDL 73811 (Figure 13), has been characterized which is apparently as safe as DFMO, but 100-200 fold more potent (63). Although far from attaining wonder-drug status, this compound cures even drug resistant East African trypanosomiasis and is toxic to cultured leishmanial parasites at micromolar concentrations as well, demonstrating the promise that inhibitors of this pathway hold (63).

As a key regulatory enzyme of the polyamine pathway, AdoMetDC has generated a great deal of interest from not only pharmacologists and drug designers, but biochemists as well. Beyond the pharmacologic relevance of AdoMetDC, the biosynthesis, activation, and enzymatic mechanism of these enzymes are of special interest to biochemists because this enzyme contains a covalently-linked pyruvoyl prosthetic group at its active site (64). To date, it is the only mammalian and kinetoplastid enzyme characterized which contains a covalently linked pyruvate prosthetic group (65). In addition, all eukaryotic AdoMetDC enzymes yet characterized are comprised of a dimer of two α- and β-subunits, which are generated by a unique post-translational cleavage of the proenzyme at a specific glutamyl-serine bond (64,66,67). As the proenzyme is processed to the active form, the N-terminal serine of the α-subunit is oxidatively deaminated to a pyruvate, which remains covalently attached at the active site (66).

From a pharmacologic standpoint it is of interest whether there is a difference in the stability of the human and parasite AdoMetDC enzymes, similar to those seen for ODC, which can also exploited in drug design schemes. It is already known that the half-life of the human AdoMetDC is approximately one hour (65). The $t_{1/2}$ for trypanosomal AdoMetDC was previously not known but has been determined to be almost 4-fold greater than its human counterpart and could therefore play a role in the trypanocidal specific activity of AdoMetDC inhibitors. It is of additional pharmacologic significance that trypanosomes and *Leishmania* contain a redox defense system which is based on trypanothione reductase and the spermidine derivative trypanothione (52). Because trypanothione contains a spermidine molecule, the synthesis of trypanothione can by blocked

by inhibition of ODC and AdoMetDC, thereby subverting the parasite's oxidant protection system while leaving that of the host intact (33,35,36). Further, it has been hypothesized that combination chemotherapy with inhibitors of the polyamine biosynthetic pathway and molecules capable of causing oxidative stress in trypanosomes, such as the 5-nitroimidazoles, could shorten the duration of curative therapies and decrease the dosages necessary to effect cures (68), although controlled clinical trials have yet to be performed.

5. Inhibitors of S-Adenosylmethionine Decarboxylase

The kinetoplastid flagellates lack a *de novo* purine biosynthetic pathway forcing *Leishmania* and the trypanosomes to transport and salvage host purines (69). The parasite transporter offers a unique opportunity to deliver toxic substrates preferentially to the pathogen (70). This is of particular importance in the treatment of African trypanosomiasis with inhibitors of AdoMetDC, since the most effective trypanocidal compounds are purine analogs that resemble decarboxylated AdoMetDC and which appear to be preferentially accumulated by the parasite (71).

This also highlights a key difference between *Leishmania* and the African trypanosomes. The delivery of toxic metabolites to leishmanial parasites is complicated by their residence within the phagocytic vacuole of the macrophage. Antiparasitic compounds must therefore not only traverse the outer membrane of the parasite, as in trypanosomes, but must also penetrate through the macrophage outer membrane as well as the lysosomal membrane. This poses a significant hurdle to the rational design of inhibitors of the leishmanial AdoMetDC if they are to function by parasite-specific purine uptake, e.g., the

proposed mechanism of MDL 73811 action. One possible approach to circumvent this problem involves the inhibition of AdoMetDC activity with spermidine analogs. The leishmanial AdoMetDC enzyme is strongly inhibited by micromolar concentrations of spermidine (see Results) and since both human and leishmanial cells transport polyamines (61) this could provide the basis of a rational chemotherapeutic regimen. Spermine analogs for the experimental therapy of human malignancies have already been developed that are inhibitory to the human AdoMetDC at micromolar concentrations, namely bis(ethyl)spermidine - $CH_3CH_2NH(CH_2)_3NH(CH_2)_4NHCH_2CH_3$, bisethylspermine - $CH_3CH_2NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCH_2CH_3$, and bisethylhomospermine - $CH_3CH_2NH(CH_2)_4NH(CH_2)_4NH(CH_2)_4NHCH_2CH_3$ (72), but have yet to be tested for their effects on leishmaniasis.

In summary, substantial evidence exists that the AdoMetDC enzyme is a promising chemotherapeutic target in the African trypanosomes and *Leishmania*, whether by direct inhibition of AdoMetDC with adenosylmethionine analogs, as demonstrated by the trypanocidal action of MDL 73811, or by the indirect allosteric inhibition of its activity through the use of spermidine analogs, e.g., the experimental antiproliferative agent bis(ethyl)spermidine. Thus, it is hoped that the molecular and biochemical characterization of the differences between the host and parasite enzymes will lead to novel and more effective approaches towards the treatment of trypanosomiasis and possibly leishmaniasis as well.

D. Drug Resistance

1. Background and Significance of Drug Resistance

Over the past half century the development of safe and effective drugs to deal with microbial and protozoal infections has literally revolutionized medical treatment and significantly decreased mortality and morbidity associated with infectious disease (22). Advances in the treatment of many malignancies has also greatly improved the outcomes of the treatments for a multitude of proliferative diseases (73).

Unfortunately, these advances in chemotherapy are already being matched by the evolution of mechanisms to circumvent drug toxicity, and drug resistance has now become a major obstacle to the successful treatment of parasitic diseases as well as those caused by bacteria, virions, and malignancies (73-75). For *Leishmania* the last decade has not only seen significant increases in the drug dosages necessary to cure leishmanial infections, but treatment failures as a result of resistance to antimonial compounds as well (76,77). Likewise, resistant strains of *T. b. rhodesiense* and *T. b. gambiense* already complicate the treatment of trypanosomiasis in Africa. Unlike the situation for leishmaniasis, however, where amphotericin B can be used as a backup in treatment failures, there are no alternative therapeutic regimens for the treatment of drug resistant sleeping sickness in endemic countries (19,78).

Drug resistance is, however, not always the foe. In the past, the development of drug resistant mutants has been of great utility in characterizing both drug metabolism and transport, as well as clarifying the targets of drug action (42,79,80).

2. Mechanisms of Drug Resistance

A basic scientific understanding of the mechanisms involved in the emergence and maintenance of drug resistance is necessary both to prevent the development of resistance and to overcome it once it has developed. Three fundamental mechanisms of drug resistance are currently recognized in eukaryotic organisms: changes in membrane permeability or the intracellular accumulation of compounds (81), decreased binding affinity via alteration of the proteins which are the chemotherapeutic target, and increased levels of the target protein (82,83).

Gene amplification as a mechanism of producing increased levels of protein occurs readily in the kinetoplastid flagellates. For example laboratory induced resistance to DFMO in *L. donovani* has been shown to result from amplification of the ornithine decarboxylase gene (84). In addition, gene amplification events have produced multiple-drug resistant phenotypes associated with the expression of the P-glycoprotein, which extrudes toxic compounds from the cell at the expense of ATP (85). As these mutants are characterized at the genomic and biochemical level, more will become known about the actual molecular mechanisms underlying the development of drug resistance.

E. The PEST Hypothesis

A fundamental tenet of molecular biology which has emerged from studies on protein targeting is that specific portions of proteins can code for their ultimate destination within the cell. The selective degradation of proteins with short half-lives can be regarded in some ways as the targeting of a protein "out of existence" and it has been proposed that certain

regions of amino acids known as PEST sequences serve as the signal for degradation (86). PEST sequences correspond to stretches of amino acids rich in proline, glutamate, serine, and threonine residues and have been demonstrated to be important for many proteins with short half-lives (87). The human and trypanosomal ornithine decarboxylase enzymes have very different half-lives, 10 minutes and 6 hours respectively, yet are very similar at the amino acid level, 62% identity, except for the presence of a carboxy-terminal sequence rich in PEST residues (58). It was recently shown that the carboxy-terminal PEST sequence of the human ODC enzyme does indeed encode its rapid degradation in mammalian cells (87). Since the mammalian ODC enzyme is stable in transfected trypanosomes, it is has been proposed that trypanosomes and *Leishmania* lack cellular proteolytic machinery present in mammals which degrades proteins with PEST sequences (88). Thus, the human and trypanosomal ODC enzymes have become a model system for elucidating the role of PEST sequences in regulating protein turnover. Along these same lines, both the mammalian and parasite AdoMetDC enzymes have PEST sequences, but still differ greatly in their enzymatic half-lives (65). Before this work it had been postulated that the leishmanial and trypanosomal AdoMetDC enzymes had longer half-lives than the human enzyme due to truncation of this carboxy-terminal PEST sequence. These data appear to support the hypothesis that these protozoa lack a selective targeting system for the rapid degradation of intracellular proteins.

THESIS RATIONALE

Although the definitive biologic roles of putrescine and the polyamines, spermidine and spermine, have yet to be determined, it is universally accepted that they are important regulators of growth and differentiation in a wide variety of cell types, including the parasitic protozoa (61), and that the inhibition of their synthesis is cytostatic. DFMO is an enzyme-activated inhibitor of ODC which has been shown to be a particularly effective drug in the treatment of the West African form of African sleeping sickness, caused by *T. b. gambiense*, by virtue of its ability to deplete the parasites of their polyamines (33,89). Unfortunately, the East African form of African sleeping sickness, caused by *T. b. rhodiesiense*, has proven to be refractory to treatment with DFMO, as is *Leishmania*, and often to other first line drugs such as suramin and the arsenicals, as well, leaving physicians with no established alternative treatments (57,90).

Inhibition of AdoMetDC, another key regulatory enzyme in the polyamine pathway, similarly obstructs the biosynthesis of the polyamines in these protozoan parasites (49,66). Inhibitors of AdoMetDC activity have recently been shown to cure a number of protozoal infections including African trypanosomiasis (71,91). More specifically, MDL 73811 (an analog of decarboxylated adenosylmethionine), has shown the ability to cure even drug resistant *T. brucei rhodiesiense* infections in mice (63). Thus, inhibitors of AdoMetDC and drugs which act on the polyamine pathway show great promise as antiparasitic targets and a firm scientific foundation exists to encourage the further study of this enzyme and the

polyamine pathway as rational chemotherapeutic targets in *Leishmania* and the African trypanosomes.

Unfortunately the trypanosomes and especially *Leishmania* contain relatively small amounts of AdoMetDC enzyme and it has proven to be virtually impossible to purify the native enzymes by conventional methods (92). The development of additional enzymatic inhibitors would therefore be greatly facilitated by an in-depth knowledge of the regulation of the AdoMetDC enzymes, the polyamine biosynthetic pathway, and the ability to purify the parasite AdoMetDCs in large quantities for biochemical, structural, and drug screening studies.

SPECIFIC AIMS

1) To identify and sequence the *AdoMetDC* gene from *L. donovani*

2) To identify and sequence the *AdoMetDC* gene and corresponding cDNA from *T. brucei*

3) To characterize the gene and the mature transcript of the *L. donovani* and *T. brucei AdoMetDC*

4) To express the recombinant *L. donovani* and *T. brucei AdoMetDC* genes in *E. coli*

5) To characterize the kinetic parameters of the recombinant *T. brucei* and *L. donovani* AdoMetDC enzymes and to measure the enzymatic half-lives of the native trypanosomal and leishmanial enzymes

6) To characterize the MDL 73811 resistant *L. donovani* cell line and to employ the *AdoMetDC* gene as a molecular probe to analyze the mechanism of drug resistance in the MDL 1000 cell line

SUMMARY OF RESULTS

1) To identify and sequence the *AdoMetDC* gene from *L. donovani*

An important aim of this thesis was the isolation and characterization of the *L. donovani AdoMetDC* gene (*AdoMetDC*) for several reasons. First, little is known about the regulation of the AdoMetDC enzyme and the polyamine biosynthetic pathway in *Leishmania*. Second, the knowledge of the amino acid sequence of *L. donovani* was a key step in the identification of the *T. brucei AdoMetDC*, an important goal which we had been unable to accomplish by cross-hybridization to the human cDNA. Third, the recombinant expression of the mammalian *AdoMetDC* in other labs had proven to be difficult (93) and by attempting to express two different parasite *AdoMetDC* genes in *Escherichia coli* (*E. coli*) one would essentially have twice the likelihood of success.

Initial attempts to isolate the *L. donovani AdoMetDC* via cross-hybrization to the human *AdoMetDC* cDNA were unsuccessful (this is now understandable in light of the 27% identity between the amino acids sequences of the enzymes) and we sought to amplify a region of the *L. donovani AdoMetDC* by the polymerase chain reaction (PCR) technique using complementary oligonucleotide primers designed to conserved regions of the AdoMetDC gene as determined from the alignment of the human and *S. cerevisiae* protein sequences. Because PCR amplified multiple segments of DNA of the appropriate length, a third oligonucleotide primer was designed and used to perform nested priming experiments on the isolated DNA segments. A 720 bp DNA segment, which amplified the correct nested PCR product, was ligated into Bluescript and sequenced to confirm the identity of the amplified fragment. This fragment was employed as a probe to isolate the

full length AdoMetDC gene from a λGEM-11 genomic library, a derivative of EMBL3. DNA was isolated from the plaque purified phage and subsequently digested with multiple restriction enzymes. By Southern hybridization analysis it was determined that a 5.1 kb *Sal*I fragment contained the full-length copy of the *AdoMetDC* gene. The *Sal*I fragment was ligated into the Bluescript KS+ plasmid and the protein coding regions, as well as 400 bp and 200 bp of the 5'- and 3'- flanking untranslated regions of the gene, respectively, were sequenced (figures 1 and 2).

2) To identify and sequence the *AdoMetDC* gene and corresponding cDNA from *T. brucei*

Another important specific aim of this thesis involved the isolation of the *T. brucei AdoMetDC*. Attempts to isolate the *T. brucei AdoMetDC* by cross-hybridization to the *L. donovani AdoMetDC* were unsuccessful even at very low stringencies. This is a common problem encountered with genes from these organisms because the leishmanial genome has a gaunosine-cytosine codon bias (94) while trypanosomes tend to have an adenosine-thymidine codon bias. Thus we were again forced to rely on a PCR-base strategy (part a).

After isolation of the *T. brucei AdoMetDC*, the availability of a complete trypanosomal cDNA library allowed us to ask two further questions: 1. does the *T. brucei AdoMetDC* mRNA have a second open reading frame similar to the human and rat *AdoMetDC* messages (part b), and 2) does *cis*-splicing occur during the maturation of the *T. brucei AdoMetDC* mRNA (part c).

a) The oligonucleotide primers used to amplify a segment of the *L. donovani AdoMetDC* did not successfully amplify the corresponding segment from *T. brucei* gDNA,

despite the use of much less stringent primer annealing conditions, e.g., 20°C. Likewise, oligonucleotide primers that had been redesigned to take advantage of the amino acid sequence homology between *L. donovani* and the human and yeast enzymes were also unsuccessful. To obtain further *AdoMetDC* sequence information in order to design more homologous oligonucleotide primers, we amplified a corresponding segment of the *Trypanosoma cruzi (T. cruzi) AdoMetDC*. *T. cruzi* is a parasitic protozoan which is generally regarded as being evolutionarily midway between *T. brucei* and *L. donovani*. This PCR product was then subcloned and sequenced, and based on this new information we were further able to modify our primer design. Because sequence information at the primer site of the PCR product could not be deduced, and this information was crucial to optimizing the primer design, we sought to amplify the 5'-end of the mature *T. cruzi* transcript by PCR using the spliced-leader sequence as the forward primer and an oligonucleotide complementary to a 3' protein coding region as the reverse primer (See specific aim 3.c.). A template for PCR was made by synthesizing cDNA from mRNA isolated from exponentially growing cultures of *T. cruzi* parasites. The PCR product, corresponding to the 5' end of the mature transcript of the *T. cruzi AdoMetDC*, was then subcloned and sequenced. With the added amino acid sequence information at the 5' primer site it was finally possible to design an oligonucleotide primer that successfully amplified a 720 bp fragment from *T. brucei* gDNA which was subsequently subcloned and sequenced to verify accurate amplification. The 717 bp fragment was employed as a probe to identify the full-length *AdoMetDC* gene in an EMBL3 genomic library. The purified phage DNA was cut with multiple restriction endonucleases and a 1.5 kb *Sal*I-*Hinc*II fragment which

encompassed the entire protein coding region of the gene was identified by Southern hybridization. The *Sal*I-*Hinc*II fragment was subsequently ligated into Bluescript KS+ and the protein coding regions as well as 295 bp of the 5'- and 132 bp of the 3'- untranslated region were sequenced (figures 3, 4, and 5).

b) In both the human and rat *AdoMetDC* cDNA sequences it was found that an alternative reading frame exists that begins 9 bases after the termination codon. These reading frames could code for a protein of 124 amino acids in the human mRNA and 125 amino acids in the rat mRNA (65). Because the functional and evolutionary significance of the 3'-open reading frame has yet to be determined, we wished to investigate whether this reading frame also existed in the *T. brucei AdoMetDC* mRNA. To this end we identified the *AdoMetDC* cDNA in a λ-ZAP library using the genomic protein coding sequence as a probe. An *in vivo* excision of the plaque purified phage was performed to obtain the cDNA in the Bluescript phagemid. The entire cDNA, 1.5 kb, was sequenced and an open reading frame of 29 amino acids was found 49 bp 3' of the *AdoMetDC* termination codon. An additional open reading frame 3' of the former termination codon was found which coded for a potential protein of 34 amino acids. Neither of these open reading frames had a potential initiation methionine. In addition, neither open reading frame exhibited homology to any region of the 3'-open reading frame of the human and rat *AdoMetDC* cDNAs. We thus conclude that no such 3'-open reading frame exists in *T. brucei AdoMetDC* cDNA.

c) To date none of the genes characterized in family trypanosomatidae have

exhibited *cis*-splicing. To determine whether *cis*-splicing occurs during the maturation of the *T. brucei AdoMetDC* transcript the open reading frame of the cDNA sequence was compared to that of the genomic sequence. No deletions or amino acid changes were found in the cDNA sequence leading us to conclude that no *cis*-splicing occurs during the maturation of the *T. brucei AdoMetDC* mRNA transcript.

3) To characterize the gene and the mature transcript of the *L. donovani* and *T. brucei AdoMetDC*

Many genes in *Leishmania* and the trypanosomes are organized as tandemly repeated arrays of two or more copies at a single genomic locus or as multiple single copies at separate genomic loci (95,96). It is not currently known what effect the gene copy number has on the development of drug resistance or on the probability of a gene amplification event in response to drug pressure, but the characterization of the gene copy number of the *AdoMetDC* genes may be of significance at some later time. The gene copy number of the leishmanial and trypanosomal *AdoMetDC*s was determined by Southern blot analysis (part a).

Further, when kinetoplastid genes are present in more than one copy number their corresponding mRNAs are sometimes processed differentially and this could have a regulatory significance during different stages of the parasite's life cycle (95). The mature transcripts of the parasite *AdoMetDC*s were characterized by Northern blot analysis (part b).

Finally, for optimal recombinant gene expression in *E. coli* (specific aim 4) it is

crucial that the proper initiation methionine be adjacent to the bacterial promoter. The predicted amino acid sequence of the *L. donovani* AdoMetDC enzyme had two possible initiation methionines, methionine 1 and methionine 11. The determination of the correct initiation methionine was further confused by the fact that the methionine 11 corresponded best with initiation methionines of the aligned amino acid sequences of the *T. brucei* and *T. cruzi AdoMetDC*s. To verify the correct initiation methionine as well as to characterize the extent of the 5'-untranslated regions of the *AdoMetDC* transcripts the trans-splicing splice sites of the mature *AdoMetDC* transcripts of both *L. donovani* and *T. brucei* were characterized (part c).

a) Southern hybridization analysis - purified genomic DNA from both *L. donovani* and *T. brucei* was digested with multiple restriction endonucleases which were either non-cutters or single cutters within the respective genes. The DNA was separated via agarose gel electrophoresis and subsequently transferred to a nylon membrane. The respective AdoMetDC coding DNAs were then radiolabeled and allowed to hybridize with the corresponding nylon membranes. Analysis of the hybridization signals suggests that the *L. donovani AdoMetDC* is present as a single-copy gene in the parasite genome (figure 7). In contrast, the *T. brucei AdoMetDC* appears to be organized as a tandemly repeated array at a single genomic locus (figure 8).

b) Northern hybridization analysis - Total RNA was purified from both *L. donovani* and *T. brucei* and separated on a denaturing agarose/formaldehyde gel. The RNA was

subsequently transferred to a nylon membrane and incubated with the respective full-length radiolabeled *AdoMetDC* genes. The autoradiographs are consistent with the presence of single transcripts of 3.1 kb and 1.8 kb in *L. donovani* and *T. brucei*, respectively (figures 9 and 10).

c) Characterization of the 5' end of the mature transcripts for the *AdoMetDC* of *L. donovani* and *T. brucei* - All known organisms within the order kinetoplastidae process their mRNA in a unique fashion, attaching a 39 bp common leader sequence, called a spliced-leader sequence, via *trans*-splicing to the 5' end of all mature transcripts. This attribute can be exploited to characterize the 5' end of the mature transcripts by defining their splice-leader junction. This technique also correctly identifies the initiation methionine, which is the first methionine, in the correct open reading frame, 3' of the splice site. PCR was employed to amplify the mature *AdoMetDC* transcript from cDNA, using the 39 bp spliced-leader sequence as the 5' sense primer and an oligonucleotide complementary to a region 3' of the initiation methionine as an antisense primer. It was found that the *trans*-splicing splice sites of the *L. donovani* and *T. brucei* transcripts were 146 bp and 143 bp 5' of the initiation methionine respectively and that methionine 1 was the correct initiation methionine of the *L. donovani AdoMetDC* (figures 1 and 3).

4) To express the recombinant *L. donovani* and *T. brucei AdoMetDC* genes in *E. coli*

The most important specific aim of this thesis, excluding the cloning of the parasite *AdoMetDC* genes, was the recombinant expression of at least one of the parasite genes for

two reasons. First, due to the high degree of divergence between the mammalian, yeast, and parasite enzymes at the amino acid sequence level, it was necessary to obtain recombinant active enzyme to prove that the cloned genes did indeed encode the AdoMetDC enzymes. Second, the ability to over-produce and characterize one parasite enzyme biochemically, as well as perhaps crystallographically later, would allow the modeling of the other parasite enzyme. Because of difficulties in obtaining sufficient amounts of trypanothione reductase enzyme from *T. brucei*, a similar rationale has been employed using the trypanothione reductase enzyme for the related plant parasite, *Crithidia fasciculata*, as a model (97).

a) *L. donovani AdoMetDC* - The production of recombinant *L. donovani AdoMetDC* enzyme was accomplished by inserting the coding sequence of the gene into the pBAce bacterial expression vector. This plasmid consists of the Bluescript® phagemid plus the bacterial *phoA* promoter which has had the periplasmic signal sequence removed. Expression of foreign gene products is accomplished by growth of the transformed *E. coli* in a low phosphate induction medium. Using PCR, a *Nde*I restriction site was introduced at the initiation methionine codon of the 5' end of the *L. donovani AdoMetDC*. The 5' end was then ligated together with the 3' end in a three-way ligation into the *Nde*I/*Sal*I site of the pBAce bacterial expression vector. This strategy was necessitated by the fact that PCR amplification of the entire *AdoMetDC* of *L. donovani* was not possible. The resultant construct was then transformed into the HT551 cell line, which is deficient in AdoMetDC activity, greatly facilitating measurement and interpretation of enzymatic activity. High level expression of the recombinant AdoMetDC protein was obtained. However, all but a small

percentage was present in an insoluble form. Despite this, the specific activity of AdoMetDC in crude bacterial extracts was 50-100 fold greater than the native activity in *L. donovani* promastigote lysates (figures 11 and 13).

i. Neither the native *T. brucei* nor the *L. donovani* AdoMetDC enzymes have yet to be purified (92). With recombinant DNA technology the rapid purification of proteins which are otherwise difficult to isolate can be accomplished by the addition of a hexa- or decahistidine peptide to the carboxy or amino terminus of a protein. The hexahistidine peptide fusion protein can then be purified over a nickel agarose column. The *L. donovani AdoMetDC* expression construct was therefore further modified to include a carboxy-terminal hexahistidine peptide at the carboxy terminus. PCR was used to amplify a 332 bp fragment of the 3' end of the *L. donovani AdoMetDC* with the reverse primer which contained the complementary sequence encoding six histidines and a *Sal*I restriction site. The PCR product was digested with the *Bgl*I and *Sal*I resriction enzymes and ligated into the *Nde*I-*Sal*I digested pBAce expression vector in a three way ligation with a *Nde*I-*Bgl*I restriction digested fragment of the original *AdoMetDC* expression construct. The final construct therefore contained only the protein coding regions of the *AdoMetDC* gene plus the hexahistidine peptide. Initial results indicate that this strategy will greatly simplify the purification of the *L. donovani* AdoMetDC enzyme.

b) *T. brucei AdoMetDC* - Because of two internal *Nde*I restriction sites within the *T. brucei AdoMetDC* the initiation methionine was mutagenized via PCR to contain a 5' *Nco*I restriction site at the initiation methionine. A *Sal*I site was likewise created 3' of the termination codon. Unfortunately, this construct has produced only low levels of recombinant protein in the *E. coli* HT551 strain, roughly equivalent to 2-5% of the AdoMetDC activity seen in *T. brucei* lysates (figure 12). Towards the goal of increasing the level of recombinant expression, a number of strategies were employed.

i. A construct was made consisting of the *T. brucei AdoMetDC* in the *Nco*I-*Bgl*I cloning site of an expression plasmid with a T5 bacteriophage promoter. The levels of expression obtained with this system corresponded to approximately 1% of the activity found in parasite lysates.

ii. A construct was made consisting of the PET expression plasmid and the *T. brucei AdoMetDC* in a *Nco*I-*Sal*I site. This plasmid directs expression via the T7 bacteriophage promoter and is somewhat unique in that 3' of the promoter is a signal sequence, the PelB leader sequence, that encodes for bacterial export of the protein into the periplasmic space. For many eukaryotic proteins this is advantageous, since in some cases it can promote proper folding. Unfortunately, in the case of the *T. brucei AdoMetDC*, this construct proved extremely toxic to all of the cell lines used and was unstable. This expression vector system could possibly work if the PelB leader sequence were removed, which is possible if a *Nde*I restriction site is used at the initiation methionine.

iii. Finally, a baculoviral expression system was employed, which is a eukaryotic expression system that employs a shuttle vector with a powerful viral promoter in conjunction with a cultured insect cell line that recognizes the viral promoter. In the past this system has been extremely successful in increasing the expression levels of eukaryotic genes whose products are mostly insoluble in bacteria. High levels of recombinant protein production were obtained with this system; however, the protein was approximately 10 kDa larger than expected and was not processed to the active α- and β-subunits. Thus, no active AdoMetDC was detected in lysates of these cells.

In brief, I consider the PET vector expression system to be the most promising mode to obtain high levels of recombinant protein. The fact that the *Nco*I-*Sal*I construct is toxic to the cells means that it is being expressed and it is likely that the trypanosomal *AdoMetDC* is similar to the *Shistosome HGPRT* which was also poorly expressed when secreted into the bacterial periplasmic space, but expressed at high levels when produced in the cytosol.

5) To characterize the kinetic parameters of the recombinant *T. brucei* and *L. donovani* AdoMetDC enzymes and to measure the enzymatic half-lives of the native trypanosomal and leishmanial enzymes

AdoMetDCs from various species show significant differences with respect to their catalytic characteristics. The *E. coli* AdoMetDC is strongly activated by Mg^{2+} and affected little by putrescine while the mammalian and yeast enzymes show no activation by Mg^{2+} but

are strongly activated by putrescine (92). In addition, a third class of AdoMetDCs apparently exists which consists of enzymes, such as those from the slime mold, *Dictostelium discoidum*, and protozoa *Tetrahymena pyriformis*, that are not activated by either putrescine or Mg^{2+} (92). It is to this third group that the kinetoplastid enzymes were initially thought to belong (62). With the isolation of the *L. donovani* and *T. brucei AdoMetDC*s it became apparent from amino acid identities with the human enzyme (figure 6) and corresponding structure function studies that both parasite enzymes would exhibit putrescine activation (part a) (Anthony Pegg: personal communication). In addition the human (65) and yeast (98) AdoMetDC enzymes are synthesized as proenzymes that are post-translationally cleaved to the α- and β-subunits of the active enzyme and we wished to determine whether the proenzyme could be detected in exponentially growing parasites (part b).

As described earlier, it has been proposed that the trypanocidal mechanism of the ornithine decarboxylase inhibitor DFMO is in part due to the differential turnover rates of the human and parasite ornithine decarboxylase enzymes. Similarly, we wished to test the hypothesis that the differential enzymatic half-lives of the human and parasite AdoMetDC enzymes also plays a role in the trypanocidal action of MDL 73811 and other inhibitors of AdoMetDC activity. Thus, it is of practical interest whether such a difference exists for the turnover rates of the host and trypanosomal AdoMetDC enzymes. Further, it is of scientific interest whether the proposed carboxy-terminal PEST sequence, which is shared by the parasite and human enzymes, leads to the rapid degradation of the parasite AdoMetDC enzymes (part c).

a) Kinetic characterization -

i. To determine the AdoMetDC K_m for S-adenosylmethionine, its natural substrate - The reaction velocity of the decarboxylation of C^{14}-S -adenosylmethionine by AdoMetDC was measured at substrate concentrations ranging from 3.1 μM to 300 μM. It was found that the recombinant *L. donovani* AdoMetDC enzyme displayed Michealis-Menton kinetics and by Lineweaver-Burk analysis of the data the K_m was determined to be 76 μM S-adenosylmethionine (figure 14). This compares well with the already established K_m of the native *T. brucei* AdoMetDC enzyme of 30 μM (62). Because of the extremely low levels of native *L. donovani* AdoMetDC activity (e.g., 1/200th of *T. brucei* levels) approximately 200 liters of cultured parasite cells would be needed to obtain a reliable K_m value for the native enzyme and these measurements were therefore not attempted.

ii. To determine effect of putrescine on the activity of the AdoMetDC enzyme - The optimal measurement of the effect of putrescine on the activity of the AdoMetDC enzyme necessitated the precipitation of the enzyme by 40% (saturation) ammonium sulfate followed by dialysis for 2-3 days against the assay buffer. The basal activity of the AdoMetDC enzyme in the absence of putrescine was found to be 4 pmol/min/mg protein. Putrescine stimulated the enzymatic activity of *L. donovani* AdoMetDC approximately three fold over a 2000-fold range of putrescine concentrations, with half-maximal

stimulation obtained at approximately 200 μM putrescine (figure 15). This is equivalent to the 3 fold putrescine activation of the dialyzed native *L. donovani* enzyme over a similar range of concentrations. In addition, the apparent half-life of the putrescine-free enzyme preparation was determined to be approximately 2 days following dialysis. In contrast to the *E. coli* enzyme, Mg^{2+} has no effect on the enzymatic activity of the leishmanial protein.

iii. To determine the effect of spermidine and spermine on the activity of the AdoMetDC enzyme and the putrescine stimulation of AdoMetDC activity - The measurement of the effects of the polyamines spermine and spermidine on *L. donovani* AdoMetDC necessitated similar enzyme preparations to those used above. Both spermidine and spermine inhibited the basal activity of AdoMetDC in the absence of putrescine by 10-25%. In addition, 2 mM spermidine and spermine ablated the maximal putrescine (2 mM) stimulation. Spermidine is the natural product of the polyamine biosynthetic pathway in the kinetoplastid parasites and measurement of the concentration dependence of the spermidine inhibition of putrescine activation showed that spermidine inhibited the activity of the AdoMetDC enzyme, in the presence of 2 mM putrescine, by 80% over a 5000-fold range of spermidine concentrations with half-maximal inhibition at 25-50 μM spermidine (figure 16).

b) Immunoblotting of *L. donovani* proteins - Lysates from stationary and exponentially growing parasite cultures were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with rabbit anti-AdoMetDC antibodies. Western blots of the stationary phase parasite proteins revealed two bands with the predicted molecular weights of the α- and β-subunits of the active enzyme (figure 18) while blots of the exponentially growing parasites showed three signals, namely two which were equivalent to the α- and β-subunits and a third with the apparent molecular weight of the proenzyme (figure 19). This suggests that the leishmanial AdoMetDC is similar to the human and yeast proteins in that it is also synthesized as a proenzyme.

c) Characterization of the enzymatic half-lives of the *T. brucei* and *L. donovani* AdoMetDC enzymes -

i. *T. brucei* turnover rate - 500 ml of exponentially growing *in vitro* culture of procyclic (TREU667) parasites were grown to a density of 1 X 10^7 cells/ml and incubated with 500 μg/ml of cycloheximide, a potent inhibitor of protein synthesis which is cytotoxic at this concentration. Aliquots of 50 ml were assayed at 0, 1, 2, 4, 8, and 24 hours for AdoMetDC activity. The results indicate that the $t_{1/2}$ of the *T. brucei* AdoMetDC enzyme is between three and four hours (figures 20 and 21) . This is approximately four-fold greater than the human AdoMetDC turnover rate of less than one hour (65).

ii. *L. donovani* AdoMetDC turnover rate - Measurement of the *L. donovani* AdoMetDC turnover rate was accomplished by a protocol similar to that for *T. brucei* above, except that a starting volume of 1 liter of cells was used, each aliquote contained 200 mls of cells, the radioactive C^{14}-S -adenosylmethionine was not diluted with non-labeled S-adenosylmethionine, and the length of the reaction time was increased from two to four hours. The results indicate that the $t_{1/2}$ of the *L. donovani* AdoMetDC enzyme is greater than 6 hours (figure 22).

iii. Cycloheximide inhibition of protein synthesis - To determine whether cycloheximide incubation was effective in eliminating protein synthesis in *T. brucei* and *L. donovani* [^{35}S]methionine-cysteine (17 μCi/ml final concentration) was added to two 15 ml aliquots of the above culture. Addition of the radioactive amino acids was delayed for ten minutes after addition of cycloheximide to one of the aliquots. At various time points 1 ml was removed and processed. The incorporation of [^{35}S]-methionine-cysteine inhibited by over 99% the cycloheximide-treated cells as opposed to the non-treated cells (figures 20 and 22), indicating that this cycloheximide concentration is sufficient to inhibit protein synthesis.

6) To characterize the MDL 73811 resistant *L. donovani* cell line and to employ the *AdoMetDC* gene as a molecular probe to analyze the mechanism of drug resistance in the MDL 1000 cell line

MDL 73811 is an experimental antiparasitic drug that is purported to be an enzyme-activated, irreversible inhibitor of the AdoMetDC enzyme and has offered a promising approach to the treatment of a number of parasitic diseases including African trypanosomiasis. Although the proposed mechanism of polyamine depletion appears straight forward there is still disagreement as to the exact growth inhibitory mechanism of the drug and it is still possible that other enzymes are inhibited by MDL 73811 (59). In the past the development of drug resistant cell lines has proven to be helpful in elucidating the molecular actions of therapeutically relevant antiparasitic compounds (79,80). Thus, to further characterize the molecular action of MDL 73811 we generated a mutant of the wild-type *L. donovani* DI700 cell line by growing the cultured parasites in sequentially increasing concentrations of MDL 73811 to a final concentration of 1000 μM (figure 23). This cell line was characterized at the molecular and biochemical level to determine if an amplification or modification of the *AdoMetDC* gene conferred the drug resistant phenotype.

The development of drug resistance in trypanosomes and *Leishmania* has been associated with a number of different mechanisms. Resistance to both DFMO and mycophenolic acid (an inhibitor of the purine salvage enzyme inosine monophosphate dehydrogenase) has been shown to result from gene amplication events of not only the gene encoding the target protein, but of extrachromosomal elements as well (99,100). To eliminate gene amplification as a mechanism of drug resistance in the MDL 1000 cell line,

Southern blot analysis of both conventional and pulsed-field gels was performed (part a). Since transcriptional and translational activation could also result in increased amounts of AdoMetDC protein, the levels of *AdoMetDC* mRNA were examined by Northern blot analysis (part b) and the AdoMetDC enzymatic activity of crude lysates of wild-type and MDL 1000 were compared (part c). To examine whether the mutant AdoMetDC enzyme was kinetically altered with respect to its sensitivity to inhibition with MDL 73811, crude lysates of both mutant and wild-type parasites were examined for AdoMetDC enzymatic activity at varied concentrations of inhibitor (part d). Kinetic alteration of membrane transport enzymes has also occurred in *Leishmania* as a mechanism of methotrexate resistance in *L. donovani*, e.g., deficiency in folate/methotrexate transport. Since MDL 73811 is a purine and is actively taken up by parasite cells (71), the mutant cell line was tested for its ability to transport other cytotoxic purine analogs (part e). Finally, to rule out the possibility that increased levels of inactive AdoMetDC enzyme were partially responsible for the drug resistance as has been demonstrated for DFMO resistant *L. donovani* parasites (60,84), Western blot analysis of SDS-PAGE separated wild-type and MDL 1000 proteins were performed (part f).

a) Southern hybridization analysis of standard and pulsed-field electrophoretically separated DNA - Genomic DNA from MDL 1000 cells and DI700 cells was digested with several different restriction endonucleases. The DNA fragments were separated via agarose gel electrophoresis and then transferred to a nylon membrane. The nylon membranes were then probed with the radioactively labeled coding sequence of the *AdoMetDC* gene. The

restriction patterns and the intensities of the hybridization signals of the DNA from the mutant cell line and the wild-type cells were equal (figure 24), ruling out the possibility of amplification of the *AdoMetDC* gene as a mechanism of drug resistance. Pulsed-field gel electrophoresis of wild-type and MDL 1000 chromosomes showed no detectable amplification nor extra chromosomal elements (figure 25).

b) Northern hybridization analysis - Total RNA was purified from the mutant and wild-type cells and separated on a denaturing agarose/formaldehyde gel. The RNA was subsequently transferred to a nylon membrane and incubated with the full length radio-labeled *AdoMetDC* gene. The autoradiograph revealed that a single transcript of 3.1 kb was present in both cell lines and that the signals were of similar intensity (figure 26), excluding the possibility of increased *AdoMetDC* mRNA as a mechanism of drug resistance.

c) Analysis of AdoMetDC activity in the MDL 1000 cell line and *L. donovani* wild-type cells - The activity of AdoMetDC was assayed in lysates of exponentially growing in vitro cultures of both wild-type and MDL 1000 mutant cells. It was found that the AdoMetDC specific activity of the mutant and the wild-type cell lines were approximately equal (figure 27).

d) Analysis of the sensitivity of the AdoMetDC enzyme of MDL 1000 and wild-type cells to inhibition with MDL 73811 - The activity of AdoMetDC was assayed in the crude lysates of exponentially growing in vitro cultures of both wild-type and MDL 1000 mutant

cells over a 1000-fold range of MDL 73811 drug concentrations. A decrease of less than 10% was observed at 1 mM MDL 73811 concentrations in both the wild-type and mutant cell lines (figure 28) indicating that the toxicity of MDL 73811 to *Leishmania* probably does not result from inhibition of the AdoMetDC enzyme.

e) Functional analysis of the purine transporter of MDL 1000 cells - Wild-type and MDL 1000 cells were assessed for their ability to transport purines by growing the cells in increasing concentrations of the toxic adenine analogs: tubercidin, puromycin, formycin B. No differences in the toxicity of these compounds was detected indicating that the resistance to MDL 73811 probably does not arise from a transport deficiency or drug extrusion mechanism, since the toxicity of other purine analogs would also be affected (figure 30).

f) Immunologic analysis of the MDL 1000 cell line with antibodies directed against the recombinant AdoMetDC protein - Antibodies were raised in rabbits directed against the recombinant AdoMetDC protein and used to probe Western blots of wild-type and mutant protein which had been separated by SDS-PAGE electrophoresis. If production of AdoMetDC was increased at the translational or post-translational level, e.g., by increasing the $t_{1/2}$, it could be detected by Western blotting. Further, if an AdoMetDC-related protein were over-produced which conveyed drug resistance by binding MDL 73811, it could possibly exhibit similar epitopes and thus be detected by the AdoMetDC antibodies. No extra or amplified signals were detected by these methods (figure 29).

In summary, we conclude that in light of the fact that the resistant phenotype of the

MDL 1000 cell does not involve any alteration in the activity, levels, or kinetic characteristics of the AdoMetDC enzyme, the antiparasitic action of MDL 73811 does not involve the AdoMetDC enzyme in *L. donovani*.

REFERENCES

1. Katz M, Despommier DD, Gwadz RW. Parasitic Diseases. 2nd ed. Heidelberg: Springer-Verlag, 1988.

2. Levinson WE, Jawetz E. Medical Microbiology and Immunology. San Mateo: Appleton and Lange, 1989.

3. Brock TD, Madigan MT. Biology of Microorganisms. 6th ed. Englewood Cliffs, NJ: Prentice Hall, 1989:546.

4. Sogin ML, Elwood HJ, Gunderson JH. Evolutionary diversity of eukaryotic *small-subunit rRNA* genes. Proceeding of the National Academy of Science USA 1986; 83:1383-1387.

5. Ou YC, Giroud C, Baltz T. Kinetoplast DNA analysis of four *Trypanosoma evansi* strains. Molecular and Biochemical Parasitology 1991; 46:97-102.

6. Hajduk S, Adler B, Bertrand K, et al. Molecular biology of African trypanosomes: development of new strategies to combat an old foe. American Journal of the Medical Sciences 1992; 303:258-270

7. Sturm NR, Degrave W, Morel C, Simpson L. Sensitive detection and schizodeme

classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. Molecular and Biochemical Parasitology 1989; 33:205-214.

8. Fairlamb AH. Future prospects for the chemotherapy of human African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 1994; 84:613-617

9. Das Gupta S, Ghosh DK, Majumder HK. A cloned kinetoplast DNA mini-circle fragment from a Leishmania spp. specific for post-kala-azar dermal leishmaniasis strains. Parasitology 1991; 102 Pt 2:187-191.

10. Grimaldi G, Jr., Momen H, Naiff RD, McMahon-Pratt D, Barrett TV. Characterization and classification of leishmanial parasites from humans, wild mammals, and sand flies in the Amazon region of Brazil. American Journal of Tropical Medicine and Hygiene 1991; 44:645-661.

11. Berman JD. Chemotherapy for leishmaniasis: biochemical mechanisms. Reviews of Infectious Diseases 1988; 10:560-586.

12. Ashford RW, Molyneux DH. The Biology of Trypanosoma and Leishmania, parasites of Man and Domestic Animals. London: Taylor and Francis, 1983.

13. de Beer P, el Harith A, Deng LL, Semiao-Santos SJ, Chantal B, van Grootheest M. A killing disease epidemic among displaced Sudanese population identified as visceral leishmaniasis. American Journal of Tropical Medicine and Hygiene 1991;44:283-289.

14. Altes J, Salas A, Riera M, et al. Visceral leishmaniasis: another HIV-associated opportunistic infection? Report of eight cases and review of the literature. AIDS 1991; 5:201-207.

15. Gramiccia M, Gradoni L, Troiani M. HIV-Leishmania co-infections in Italy. Isoenzyme characterization of *Leishmania* causing visceral leishmaniasis in HIV patients. Transactions of the Royal Society of Tropical Medicine and Hygiene 1992; 86:161-163.

16. Medrano FJ, Hernandez-Quero J, Jimenez E, et al. Visceral leishmaniasis in HIV-1-infected individuals: a common opportunistic infection in Spain? AIDS 1992; 6:1499-1503.

17. Gasser RA, Jr., Magill AJ, Oster CN, Tramont EC. The threat of infectious disease in Americans returning from Operation Desert Storm [see comments]. New England Journal of Medicine 1991; 324:859-864.

18. Grogl M, Daugirda JL, Hoover DL, Magill AJ, Berman JD. Survivability and infectivity of viscerotropic Leishmania tropica from Operation Desert Storm participants in

human blood products maintained under blood bank conditions. American Journal of Tropical Medicine and Hygiene 1993; 49:308-315.

19. Grimaldi G, Tesh RB. Leishmaniases of the New World: Current Concepts and Implications for Future Research. Clinical Microbiology Reviews 1994; 6:230-250.

20. Locksley RM. Leishmaniasis. In: Wilson JD, Braunwald E, Isselbacher KJ, et al, eds. Harrison's principles of internal medicine. 12th ed. New York: McGraw-Hill, Inc., 1991:789-791.

21. Berger BJ, Fairlamb AH. Interactions between immunity and chemotherapy in the treatment of the trypanosomiases and leishmaniases. Parasitology 1992; 105 Suppl:S71-S78.

22. Rang HP, Dale MM. Pharmacology. 2nd ed. London: Churchill Livingstone, 1991:863-865.

23. Modabber F. Development of vaccines against leishmaniasis. Scandinavian Journal of Infectious Diseases Supplementum 1990; 76:72-78.

24. Jordan WS, Jr.. Impediments to the development of additional vaccines: vaccines against important diseases that will not be available in the next decade. Reviews of Infectious Diseases 1989; 11 Suppl 3:S603-S612.

25. Ford J. The Role of Trypanosomiases in African Ecology. A Study of the Tsetse Fly Problem. Oxford: Clarendon Press, 1971.

26. Wellde BT, Chumo DA, Waema D, Reardon MJ, Smith DH. A history of sleeping sickness in Kenya. Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:1-11.

27. Mihok S, Otieno LH, Darji N. Population genetics of *Trypanosoma brucei* and the epidemiology of human sleeping sickness in the Lambwe Valley, Kenya. Parasitology 1990; 100 Pt 2:219-233.

28. Kuzoe FAS. Current situation of African trypanosomiasis. Acta Tropica 1993; 54:153-162.

29. White MW, Kameji T, Pegg AE, Morris DR. Increased efficiency of translation of ornithine decarboxylase mRNA in mitogen-activated lymphocytes. European Journal of Biochemistry 1987; 170:87-92.

30. Wellde BT, Chumo DA, Reardon MJ, et al. Epidemiology of Rhodesian sleeping sickness in the Lambwe Valley, Kenya. Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:43-62.

31. Wellde BT, Chumo DA, Reardon MJ, et al. Presenting features of Rhodesian

sleeping sickness patients in the Lambwe Valley, Kenya. Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:73-89.

32. Wellde BT, Chumo DA, Reardon MJ, et al. Diagnosis of Rhodesian sleeping sickness in the Lambwe Valley (1980-1984). Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:63-71.

33. Jennings FW. Future prospects for the chemotherapy of human African trypanosomiasis. 2. Combination chemotherapy and African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 1990; 84:618-621.

34. Wellde BT, Waema D, Chumo DA, et al. Review of tsetse control measures taken in the Lambwe Valley in 1980-1984. Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:119-125.

35. Fairlamb AH. Future prospects for the chemotherapy of human trypanosomiasis. 1. Novel approaches to the chemotherapy of trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 1990; 84:613-617.

36. Fairlamb AH. Trypanothione metabolism and rational approaches to drug design. Biochemical Society Transactions 1990; 18:717-720.

37. Schacter LP, Anderson C, Canetta RM, et al. Drug discovery and development in the pharmaceutical industry. Seminars in Oncology 1992; 19:613-621.

38. Taylor G. Drug design. A rational attack on influenza [news; comment]. Nature 1993; 363:401-402.

39. Landfear SM, Wirth DF. Structures of the mRNA encoded by tubulin genes in Leishmania enrietti. Molecular and Biochemical Parasitology 1985; 15:61-82.

40. Wang CC. Basic Principles of Antiparasitic Chemotherapy. 1990:634-644.

41. Martin YC. Computer-assisted rational drug design. Methods in Enzymology 1991; 203:587-613.

42. Hyde JE. Molecular Parasitology. New York: Van Nostrand Reinhold, 1990.

43. Ginger CD. Possibilities for new antiprotozoal drugs: the TDR/WHO approach. London: Taylor and Francis, 1991:605-621.

44. McCann PP, Pegg AE. Ornithine decarboxylase as an enzyme target for therapy. Pharmacology and Therapeutics 1992; 54:195-215.

45. Pegg AE, McCann PP. S-adenosylmethionine decarboxylase as an enzyme target for therapy. Pharmacology and Therapeutics 1992; 56:359-377.

46. Janne J, Alhonen L, Leinonen P. Polyamines: from molecular biology to clinical applications. Annals of Medicine 1991; 23:241-259.

47. Bacchi CJ, Yarlett N. Effects of antagonists of polyamine metabolism on African trypanosomes. Acta Tropica 1993; 54:225-236.

48. Coffino P, Poznanski A. Killer polyamines? Journal of Cellular Biochemistry 1991; 45:54-58.

49. Tabor CW, Tabor H, Xie Q. Spermidine synthase of *Escherichia coli*: Localization of the spe2 gene. Proceeding of the National Academy of Science USA 1986; 83:6040-6044.

50. Canellakis ZN, Marsh LL, Bondy PK. Polyamines and their derivatives as modulators in growth and differentiation. Yale Journal of Biology and Medicine 1989.

51. Balasundaram D, Tyagi AK. Polyamine--DNA nexus: structural ramifications and biological implications. Molecular and Cellular Biochemistry 1991; 100:129-140.

52. Benson TJ, McKie JH, Garforth J, Borges A, Fairlamb AH, Douglas KT. Rationally

designed selective inhibitors of trypanothione reductase. Phenothiazines and related tricyclics as lead structures. Biochemical Journal 1992; 286:9-11.

53. Bacchi CJ, Sufrin JR, Nathan HC, et al. 5'-Alkyl-substituted analogs of 5'-methylthioadenosine as trypanocides. Antimicrobial Agents and Chemotherapy 1991; 35:1315-1320.

54. Bush GL, Tassin A, Friden H, Meyer DI. Purification of a translocation-competent secretory protein precursor using Nickel ion affinity chromatography. Journal of Biological Chemistry 1991; 266:13811-13814.

55. Persson L, Stjernborg L, Holm I, Heby O. Polyamine-mediated control of mammalian S-adenosyl-L-methionine decarboxylase expression: effects on the content and translational efficiency of the mRNA. Biochemical and Biophysical Research Communications 1989; 160:1196-1202.

56. Shantz LM, Holm I, Janne OA, Pegg AE. Regulation of S-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content. Biochemical Journal 1992; 288:511-518.

57. Bacchi CJ, Nathan HC, Livingston T, et al. Differential susceptibility to DL-alpha-difluoromethylornithine in clinical isolates of *Trypanosoma brucei rhodesiense*.

Antimicrobial Agents and Chemotherapy 1990 Jun;34(6):1183 8 1990;

58. Phillips MA, Coffino P, Wang CC. Cloning and sequencing of the ornithine decarboxylase gene from *Trypanosoma brucei*. Journal of Biological Chemistry 1987; 262:8721-8727.

59. Byers TL, Bush TL, McCann PP, Bitonti AJ. Antitrypanosomal effects of polyamine biosynthesis inhibitors correlate with increases in *Trypanosoma brucei brucei* S-adenosyl-L-methionine. Biochemical Journal 1991; 274:527-533.

60. Coons T, Hanson S, Bitonti AJ, McCann PP, Ullman B. Alpha-difluoromethylornithine resistance in *Leishmania*. Molecular and Biochemical Parasitology 1990; 39:77-89.

61. Bacchi CJ, McCann PP. Parasitic Protozoa and Polyamines. In: McCann PP, Pegg AE, Sjoerdsma A, eds. Inhibition of Polyamine Metabolism: biological significance and basis for new therapies. San Diego: Academic Press, 1987:317-344.

62. Bitonti AJ, Dumont JA, McCann PP. Characterization of *Trypanosoma brucei brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(haunylhydrazone). Biochemical Journal 1986; 237:685-689.

63. Bitonti AJ, Byers TL, Bush TL, et al. Cure of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* infections in mice with an irreversible inhibitor of S-adenosylmethionine decarboxylase. Antimicrobial Agents and Chemotherapy 1990; 34:1485-1490.

64. Stanley BA, Pegg AE, Holm I. Site of pyruvate formation and processing of mammalian S-adenosylmethionine decarboxylase proenzyme. Journal of Biological Chemistry 1989; 264:21073-21079.

65. Pajunen A, Crozat A, Janne OA, et al. Structure and regulation of mammalian S-adenosylmethionine decarboxylase. Journal of Biological Chemistry 1988; 263:17040-17049.

66. Tabor CW, Tabor H. The speEeD Operon of *Escherichia coli*: Formation and processing of a proenzyme form of S-adenosylmethionine decarboxylase. Journal of Biological Chemistry 1987; 262:16037-16040.

67. Stanley BA, Pegg AE. Amino acid residues necessary for putrescine stimulation of human S-adenosylmethionine decarboxylase proenzyme processing and catalytic activity. Journal of Biological Chemistry 1991; 266:18502-18506.

68. Eze MO. Towards more efficacious chemotherapy of trypanosomiasis: combination of alpha-difluoromethylornithine (DFMO) with reactive oxygen generating drugs. Medical

Hypotheses 1991; 36:246-249.

69. Marr JJ. Purine analogs as chemotherapeutic agents in leishmaniasis and American trypanosomiasis. Journal of Laboratory and Clinical Medicine 1991; 118:111-119.

70. Fairlamb AH. Novel biochemical pathways in parasitic protozoa. Parasitology 1989; 99 Suppl:S93-112.

71. Byers TL, Casara P, Bitonti AJ. Uptake of the antitrypanosomal drug 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine (MDL 73811) by the purine transport system of *Trypanosoma brucei brucei*. Biochemical Journal 1992; 283:755-758.

72. Kawasaki ES. Amplification of RNA. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols. A Guide to Methods and Applications. San Diego, CA: Academic Press, 1990:21-27.

73. Moscow JA, Cowan KH. Multidrug resistance. Journal of the National Cancer Institute 1988; 80:14-20.

74. Cohen ML. Epidemiology of drug resistance: implications for a post-antimicrobial era [see comments]. Science 1992; 257:1050-1055.

75. Tiirikainen MI, Krusius T. Multidrug resistance. Annals of Medicine 1991; 23:509-520.

76. Grogl M, Thomason TN, Franke ED. Drug resistance in leishmaniasis: its implication in systemic chemotherapy of cutaneous and mucocutaneous disease. American Journal of Tropical Medicine and Hygiene 1992; 47:117-126

77. Herwaldt BL, Berman JD. Recommendations for treating leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. American Journal of Tropical Medicine and Hygiene 1992; 46:296-306.

78. Pepin J, Milord F, Mpia B, et al. An open clinical trial of nifurtimox for arseno-resistant *Trypanosoma brucei gambiense* sleeping sickness in central Zaire. Transactions of the Royal Society of Tropical Medicine and Hygiene 1989; 83:514-517.

79. Carter NS, Fairlamb AH. Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature 1993; 361:173-176.

80. Fairlamb AH, Carter NS, Cunningham M, Smith K. Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance toother drugs and trypanothione metabolism. Molecular and Biochemical Parasitology 1992; 53:213-222.

81. Beck J, Ullman B. Characterization of a mutant clone of *Leishmania donovani* deficient in folate transport. Advances in Experimental Medicine and Biology 1989; 253B:533-538.

82. Dever LA, Dermody TS. Mechanisms of bacterial resistance to antibiotics. Archives of Internal Medicine 1991; 151:886-895.

83. Kaur K, Coons T, Emmett K, Ullman B. Methotrexate-resistant *Leishmania donovani* genetically deficient in the folate-methotrexate transporter. Journal of Biological Chemistry 1988; 263:7020-7028.

84. Hanson S, Adelman J, Ullman B. Amplification and molecular cloning of the *ornithine decarboxylase* gene of *Leishmania donovani*. Journal of Biological Chemistry 1992; 267:2350-2359.

85. Henderson DM, Sifri CD, Rodgers M, Wirth DF, Hendrickson N, Ullman B. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene homologous to the mammalian mdr1 gene. Molecular and Cellular Biology 1992; 12:2855-2865.

86. Rogers S, Wells R, Rechsteiner M. Amino Acid Sequences Common to Rapidly Degraded Proteins: The PEST Hypothesis. Science 1986; 234:364-369.

87. Loetscher P, Pratt G, Rechsteiner M. The C terminus of mouse ornithine decarboxylase confers rapid degradation on dihydrofolate reductase. Journal of Biological Chemistry 1991; 266:11213-11220.

88. Bass KE, Sommer JM, Cheng QL, Wang CC. Mouse ornithine decarboxylase is stable in Trypanosoma. Journal of Biological Chemistry 1992; 267:11034-11037.

89. Van Voorhis WC. Therapy and prophylaxis of systemic protozoan infections. Drugs 1990; 40:176-202.

90. Wellde BT, Chumo DA, Reardon MJ, et al. Treatment of Rhodesian sleeping sickness in Kenya. Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:99-109.

91. Bacchi CJ, Nathan HC, Yarlett N, et al. Cure of murine *Trypanosoma brucei rhodesiense* infections with an S-adenosylmethionine decarboxylase inhibitor. Antimicrobial Agents and Chemotherapy 1992; 36:2736-2740.

92. Tekwani BL, Bacchi CJ, Pegg AE. Putrescine activated S-adenosylmethionine decarboxylase from *Trypanosoma brucei brucei*. Molecular and Cellular Biochemistry 1992; 117:53-61.

93. Trypanosomiasis in the Lambwe Valley, Kenya. Annals of Tropical Medicine and Parasitology 1989; 83 Suppl 1:1-220.

94. Langford CK, Ullman B, Landfear SM. Leishmania: codon utilization of nuclear genes. Experimental Parasitology 1992; 74:360-361.

95. Allen TE, Ullman B. Cloning and expression of the *hypoxanthine-guanine phosphoribosyltransferase* gene from *Trypanosoma brucei*. Nucleic Acids Research 1993; 21:5431-5438.

96. Hendrickson N, Allen T, Ullman B. Molecular characterization of *phosphoribosylpyrophosphate transferase* gene of *Leishmania donovani*. Molecular and Biochemical Parasitology 1993; 59:15-27.

97. Henderson GB, Yamaguchi M, Novoa L, Fairlamb AH, Cerami A. Biosynthesis of the trypanosomatid metabolite trypanothione. Biochemistry 1990; 29:3924-3929.

98. Kashiwagi K, Taneja SK, Liu TY, Tabor CW, Tabor H. Spermidine biosynthesis in Saccharomyces cerevisiae. Biosynthesis and processing of a proenzyme form of S-adenosylmethionine decarboxylase. Journal of Biological Chemistry 1990; 265:22321-22328.

99. Hanson S, Beverley SM, Wagner W, Ullman B. Unstable amplification of two

extrachromosomal elements in alpha-difluoromethylornithine-resistant *Leishmania donovani*. Molecular and Cellular Biology 1992; 12:5499-5507.

100. Wilson K, Collart FR, Huberman E, Stringer JR, Ullman B. Amplification and molecular cloning of the *IMP dehydrogenase* gene of *Leishmania donovani*. Journal of Biological Chemistry 1991; 266:1665-1671.

Cloning and Functional Expression of the S-adenosylmethionine Decarboxylase Gene of *Leishmania donovani* and *Trypanosoma brucei*

by

Jerry Scott and Buddy Ullman

Department of Biochemistry and Molecular Biology

Oregon Health Sciences University

Portland, Oregon 97201-3098

Telephone: (503) 494-8437 FAX: (503) 494-8393

SUMMARY

The polyamine biosynthetic pathway is an important target for antiparasitic chemotherapy as demonstrated by the remarkable success of difluoromethylornithine (DFMO) in the treatment of West African sleeping sickness, caused by *Trypanosoma brucei gambiense* (*T. b. gambiense*). Despite these successes efficable drug therapies for the more refractory East African form and for a related protozoal infection, visceral leishmaniasis, remain important goals. A recently discovered inhibitor of S-adenosylmethionine decarboxylase (AdoMetDC) has demonstrated great promise in the treatment of murine models of East African trypanosomiasis as well as being toxic to cultured *Leishmania donovani* (*L. donovani*) parasites. Thus, the parasite AdoMetDC enzymes could represent a possible chemotherapeutic target. To characterize the *T. brucei* and *L. donovani* AdoMetDC enzymes in detail the coding sequence of the *T. brucei* and *L. donovani* *AdoMetDC*s have been isolated from genomic libraries by cross-hybridization to their respective partial-length PCR products, amplified using sequence homology between the human and yeast enzymes. The *T. brucei* and *L. donovani* clones encompassed a 1.5 kb *Sal*I-*Hinc*II and a 5.1 kb *Sal*I fragment respectively and contained single open reading frames of 1,110 bp and 1,176 bp, encoding proteins of 370 and 392 amino acids each. Maximum gap alignment revealed that the predicted amino acid sequence of the parasite AdoMetDCs were 22% and 27% identical to the AdoMetDC proteins of the yeast and human enzymes, respectively, and shared essentially no homology with the *E. coli* enzyme. Despite this lack of overall homology the *T. brucei* and *L. donovani* enzymes are similar to other eukaryotic

AdoMetDCs in that they appear to be synthesized as a proenzyme that is cleaved at a glutamyl-serine bond to produce the α- and β-subunits of the active enzymes. Thus, the cleavage sites of the proenzymes are identical to the cleavage sites of other eukaryotes, with high conservation of the surrounding eight amino acids. Southern blot analysis of the genomic DNAs revealed that the *T. brucei AdoMetDC* is present in multiple copies, which appear to be tandemly repeated, while the *L. donovani* gene is present as a single copy. Analysis of *T. brucei* and *L. donovani* mRNA by Northern blotting revealed that the coding sequence of the *AdoMetDC* genes hybridized to single 1.8 kb and 3.1 kb transcripts, respectively. The recombinant genes were expressed in the *AdoMetDC*-deficient *E. coli* cell line, HT551, using the pBAce bacterial expression vector. The recombinant *T. brucei AdoMetDC* expressed poorly; however, high levels of expression were obtained for the recombinant *L. dovovani AdoMetDC*, ranging from 40 - 60% of the total cellular protein. Although most of the recombinant *L. donovani* protein was present in the insoluble fraction, in the form of inclusion bodies, a significant amount was soluble, corresponding to between 50 and 100-fold greater levels of AdoMetDC activity (3 nmol of CO_2/h/mg of protein) than in promastigote *L. donovani* lysates and was sufficient to allow the biochemical characterization of the enzyme. The recombinant enzyme had a calculated K_m for S-adenosylmethionine of 76 μM. The activity of the leishmanial AdoMetDC enzyme was stimulated approximately three fold over basal levels by the presence of 2 mM putrescine with half-maximal stimulation at 50 μM. The putrescine stimulation of activity was strongly inhibited by micromolar concentrations of spermidine. Rabbit antibodies were raised against the insoluble fraction of the recombinant *L. donovani* protein and were used to

probe Western blots of SDS-PAGE separated promastigote parasite proteins. Western blots of parasites harvested during stationary phase showed two bands with the apparent molecular weights of the α- and β-subunits of the active enzyme, while Western blots of exponentially growing parasite proteins revealed a third band with the molecular weight of the proenzyme.

To further characterize the chemotherapeutic mechanism of the experimental drug MDL 73811, an MDL 73811 resistant, *L. donovani* derived, cell line was generated by growing the parasites in sequentially increasing concentrations of the drug until a mutant was obtained capable of growing at concentrations which were 250-fold greater than lethal concentrations for wild-type cells. The *AdoMetDC* gene was used to characterize the mutant cell line at a molecular level and both Southern and Northern analysies indicate that no amplification or transcriptional activation of the *AdoMetDC* gene has occurred. Biochemical studies showed essentially no difference in the specific activity of AdoMetDC of the cell lines. Likewise the AdoMetDC activity of both cell lines was affected little by millimorlar concentrations of MDL 73811. It is, therefore, likely that the growth inhibitory effects of MDL 73811 in *L. donovani* do not result from inhibition of the AdoMetDC enzyme.

INTRODUCTION

The available therapeutic regimens for parasitic diseases trail far behind the significant advances made in the chemotherapy of other infectious organisms. More specifically, the therapies for African sleeping sickness and visceral leishmaniasis, two devastating and often fatal diseases caused by the protozoan parasites *T. brucei* and *L. donovani*, have changed little in the last fifty years, and both are now sometimes complicated by resistance to the traditional treatment modalities (1-3). The only newly discovered compound to reach clinical use, difluoromethylornithine (DFMO), is ineffective for the treatment of many isolates of East African trypanosomes, e.g., *T. b. rhodesiense* (4), and has little efficacy in treating the related intracellular parasite, *L. donovani* (5). The rational design of novel chemotherapeutic agents would be greatly facilitated by a better understanding of the unique biochemistry and biosynthetic pathways in these organisms.

The concentrations of putrescine and the polyamines, spermidine and spermine, are greatly increased in actively dividing cells and the inhibition of their synthesis is cytostatic to eukaryotic cells making this pathway an attractive target for the chemotherapy of neoplastic as well as parasitic diseases (6). The polyamine spermidine has, however, an added significance in *Leishmania* and the trypanosomes in that spermidine is obligately incorporated into a unique and indispensible redox-defense molecule known by the trivial name trypanothione (7). Trypanothione is the parasite equivalent of the glutathione molecule of higher eukaryotes, thereby making the inhibition of polyamine biosynthesis not only cytostatic to these organisms but subverting their redox defenses as well (8). Further,

parasite-specific inhibition of the polyamine biosynthetic pathway has already proven to be effective in the treatment of West African trypanosomiasis, through the FDA approved therapeutic use of DFMO, a suicide-substrate of ornithine decarboxylase, an obligatory enzyme in the polyamine pathway (3)..

S-adenosylmethionine decarboxylase (AdoMetDC) [EC 4.1.1.50], like ornithine decarboxylase, is an indispensible enzyme in the biosynthesis of spermidine in *Leishmania* and the trypanosomes, as well as representing a significant regulatory element of the polyamine pathway. A recently discovered enzyme-activated inhibitor of AdoMetDC, MDL 73811, has shown great promise in the treatment of murine models of trypanosomiasis, displaying approximately 100-fold greater parasite-specific toxicity than DFMO and curing even the more fulminant East African form of the disease (9).

Previous biochemical studies of the *T. brucei* AdoMetDC enzyme have shown that it could differ significantly from the human enzyme (10,11); however, the AdoMetDC enzyme from *L. donovani* has yet to be characterized and little is known concerning the regulation of the polyamine biosynthetic pathway in *Leishmania*. The biochemical characterization of the parasite AdoMetDC enzymes has been hindered by the lack of a suitable method for their purification and by the low levels of AdoMetDC activity present in *Leishmania*. Thus, the biochemical characterization of the trypanosomal and leishmanial AdoMetDC enzymes would be greatly simplified and our understanding of their role in the control of the polyamine biosynthetic pathway furthered by the cloning of the parasite *AdoMetDC* genes and the ability to produce recombinant enzyme.

MATERIALS AND METHODS

Chemicals and Reagents - [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{35}S]dATP (1320 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [^{14}C]Hypoxanthine (56 mCi/mmol), [^{14}C]guanine (56 mCi/mmol), and [^{14}C]xanthine (56 mCi/mmol) were bought from Moravek Biochemicals (Brea, CA). [^{14}C]HPP (46 mCi/mmol) was obtained from Research Products International Corp. (Mount Prospect, IL). Synthetic oligonucleotides were prepared by OLIGO'S ETC. (Wilsonville, OR). Thermostable AmpliTaq DNA polymerase was obtained from the Perkin-Elmer Corp. (Norwalk, CT). All restriction and DNA modifying enzymes were acquired from either Bethesda Research Laboratories Life Technologies Inc. (Gaithersburg, MD) or Boehringer Mannheim Biochemicals (Indianapolis, IN). All other materials, chemicals, and reagents were of the highest quality commercially available.

Cell Culture - L. donovani promastigotes were grown in the completely defined Dulbecco's modified Eagle- Leishmania (DME-L) culture medium (12). Promastigotes were cultivated continuously in a humidified 10% CO2 atmosphere at 26 C. The DI700 clone of the 1S Sudanese strain was used in all experiments involving *Leishmania.*

Procyclic forms of the pleiomorphic *T. b. brucei* TREU667 strain were propagated in SDM-79 medium (13) supplemented with 10% heat-inactivated fetal calf serum purchased from HyClone Inc. (Logan, UT).

Nucleic acid Isolation - Genomic DNA was purified from cultured parasites, either 1.0X10^{10} procyclic *T. brucei* cells or an equal number of *L. donovani* promastigote cells, as described (14). Total RNA was prepared by the phenol-chloroform extraction

protocol reported by Landfear and Wirth (15).

Isolation of a L. donovoani AdoMetDC Gene Fragment by PCR - To generate a homologous DNA probe for the isolation of the *L. donovani AdoMetDC*, a fragment of the gene was amplified from genomic DNA via PCR using degenerate oligonucleotide primers created from conserved amino acid sequences of the mammalian (16) and yeast (17) AdoMetDC proteins. A sense primer, 5'-CTCGGAATTCC-TT[CT]-GAG-GG[CGT]-[AC]C[CG]-GAG-AA-3, was constructed with an 11 nucleotide leader encompassing an *Eco*RI site 5' to a degenerate oligonucleotide sequence corresponding to amino acids 7 - 12, FEGPEK, of the human AdoMetDC enzyme. The antisense primer, 5'-CTCGGGATCCC-CTC-[ACG]GG-[CG]GT-[AG]AT-GTG-3', was synthesized with a *Bam*HI site preceded by a 3 nucleotide leader 5' to a mixed oligonucleotide generated from residues 243 - 248, HITPEK, of the human AdoMetDC. Amplification of the specific *AdoMetDC* fragment was accomplished on a Coy Instruments (Ann Arbor, MI) thermocycler using the amplification assay mixture described by Hanson et al. with minor modifications (16). The PCR reaction was allowed to proceed through 30 cycles of denaturation at 94° for 0.5 min, annealing at 35° for 1.5 min, and extension at 72° 1.5 min. The PCR products were subsequently separated by gel electrophoresis, digested with *Bam*HI and *Eco*RI, ligated into appropriately digested pBluescript KS^+ vector from Stratagene (San Diego, CA), and transformed into XL-1 Blue *E. coli*. Large scale plasmid preparations were prepared according to the large scale alkaline lysis protocol of the Qiagen maxi-prep kit (Chatsworth, CA). The 720 bp *L. donovani AdoMetDC* fragment was sequenced according to the dideoxy chain termination method (18) with

[^{35}S]dATP as the radiolabel and the Sequenase 2.0 sequencing kit from United States Biochemical Corp (Cleveland, OH).

Isolation of a T. brucei AdoMetDC Gene Fragment by PCR - The primers used for *Leishmania* did not successfully amplify any PCR products from *T. brucei*, even with lower annealing temperatures. Attempts to isolate the *T. brucei* gene via cross-hybridization were confounded by the gaunosine-cytosine bias of the leishmanial genome (19). To circumvent this problem a corresponding segment of the *T. cruzi AdoMetDC* gene was amplified, subcloned, and sequenced to obtain added amino acid homology with which to further optimize primer design. The optimized sense primer, 5'-CTCGGAATTCC-TT[CT]-GA[GA]-GGI-CCI-GA[GA]-AA[GA]-[TC]TI-GA[GA]-GA-3', was constructed with an 11 nucleotide leader encompassing an *Eco*RI site 5' to a degenerate oligonucleotide sequence corresponding to amino acids 42 - 50, FEGPDKRLE, of the leishmanial AdoMetDC enzyme. The antisense primer, 5'-CTCGGGATCCC-[CT]TC-IGG-IGT-[AGT]AT-[AG]TG-XAT-XGT- 3', was synthesized with a *Bam*HI site preceded by a 3 nucleotide leader 5' to the degenerate oligonucleotide generated from residues 275 - 282, TIHITPE, of the leishmanial AdoMetDC. The amplification, subcloning, and sequencing of the 717 bp segment of the *T. brucei AdoMetDC* amplified by PCR were performed as described above for the *L. donovani AdoMetDC*.

L. donovani and T. brucei Genomic DNA Libraries - A genomic library of DI700 DNA was constructed by partial digestion of genomic DNA with *Sau3A* restriction endonuclease and size selecting the DNA fragments on sucrose gradients. 16-24-kb

DNA fragments were ligated into the BamHI site of the bacteriophage vector λ-GEM-11, a derivative of EMBL3, using the protocol described in the brochure provided by Promega (Madison, WI).

The *T. brucei* genomic library was generously provided by Dr. Maryln Parsons and Dr. Peter Myler and was originally prepared from the IsTAR 1 clone of the EATRO 164 strain of *T. b. brucei.*

Isolation of the AdoMetDC from L. donovani and T. brucei Genomic Libraries - 50,000 plaques from the respective *L. donovani* and *T. brucei* genomic libraries were plated, transferred to Nytran filters (Schleicher & Schuell, Keene, NH), and probed with the respective PCR-product. The prehybridization, hybridization, and washing conditions employed to isolate the respective *AdoMetDC* genomic loci were identical to those described previously by this laboratory for the isolation of the *L. donovani* ornithine decarboxylase (ODC) gene (20). The three positive bacteriophage from each initial screening were carried through a tertiary plaque purification, and their DNA was isolated by CsCl density gradient centrifugation (20).

Subcloning and Sequencing of the T. brucei and L. donovani AdoMetDC - DNA from the genomic clones was cleaved with a variety of restriction endonucleases, electrophoresed, transferred to Nytran membranes and probed with the PCR products under the same high stringency conditions employed for screening the libraries. The *L. donovani AdoMetDC* was isolated in a 5.1 kb *Sal*I genomic fragment that hybridized to the PCR product. Similarly, the *T. brucei AdoMetDC* was isolated in a 1.4 kb *Sal*I-*Hinc*II fragment. Both were ligated into the pBluescript KS^+ phagmemid and transformed into

the XL-1 Blue *E. coli* cell line. Large scale plasmid preparations of pBluescript containing the parasite DNAs and nucleotide sequencing of the DNAs were performed as described above for the PCR fragment. Double stranded sequencing was performed on the *T. brucei AdoMetDC*. Because of difficulty in obtaining sequence from certain regions of the leishmanial gene, both single stranded and double stranded sequencing were performed on the *L. donovani AdoMetDC*. Single stranded DNA was prepared from plasmids containing the *Sal*I fragment as described (23).

Computer Assisted DNA and Amino Acid Sequence Analysis - Analyses of nucleotide and predicted amino acid sequences were performed on an IBM compatible AT computer using the Sequence Analysis Program from International Biotechnologies, Inc. (New Haven, CT). Protein sequences were aligned using the CLUSTAL V multiple sequence alignment program, a modification of the CLUSTAL package described by Higgins and Sharp (21). Amino acid similarity scores between nonidentical amino acid pairs were obtained from the log-odds amino acid similarity matrix of Dayhoff (22).

Southern and Northern Hybridizations - Southern blots of genomic DNA that had been digested with the appropriate restriction enzymes using the conditions recommended by the supplier were performed as described (23). For identification of specific *AdoMetDC* transcripts total RNA was electrophoresed on 1.2% agarose gels containing 2.2 M formaldehyde (24). The Northern blots were hybridized to the radiolabeled *AdoMetDC* coding sequences under the same high stringency conditions described for probing the library screens.

Mapping the 5' Termini of the Mature AdoMetDC mRNAs from L. donovani and T.

brucei - Because of the marked difference in the predicted amino termini between the *T. brucei* and *L. donovani* AdoMetDC amino acid sequences, we sought to confirm the position of the initiation methionine by mapping the 5' termini of the mature *L. donovani* and *T. brucei AdoMetDC* transcripts. The initiation methionine of trypanosomatid transcripts is generally accepted to be the first in frame methionine codon 3' of the trans-splicing splice site. The 5' primed termini were defined by a modification of the PCR amplification protocol reported by Hanson et al. (20). The cDNA synthesis from total RNA was achieved with random hexamer primers using the reverse transcriptase (RT) protocol described by Kawasaki (25). The RT mixture was then adjusted to 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$, and 10 pmol of each oligonucleotide primer and 5 units of *Taq* DNA polymerase were added. The sense primer, 5'-

CTCGGGATCCCAACGCTATATAAGTATCAGTTTCTGTACTTTATTG - 3',

contained an 11 nucleotide leader containing a *Bam*HI restriction site followed by 31 nucleotides matching positions 5 - 35 of the *L. donovani* mini-exon (26). The mini-exon is an RNA that is *trans*-spliced onto the 5' end of all mRNAs from *Trypanosoma* and *Leishmania* species (27,28). Of the 31 nucleotides of the sense primer, the *T. brucei* and *L. donovani* mini-exon genes differ in only 4 positions, thus ensuring efficient priming of the cDNA in the PCR. The antisense primer for the leishmanial transcript, 5'-CTCGAATTCCGCGCTGATTGCCTCGAGGATGTTGGG-3', was constructed with a 9 nucleotide leader that contained an *Eco*RI recognition site 5' to nucleotides 370 - 396 of the predicted protein coding portion of the *L. donovani AdoMetDC*. The antisense

primer for the amplification of the trypanosomal transcript, 5'-CTCGAATTCCCATTCTACTTCGCCGCAAACAGT-3', was constructed with a similar 9 nucleotide leader that contained an *Eco*RI recognition site 5' to nucleotides 352 - 375 of the predicted protein coding portion of the *T. brucei AdoMetDC*. The PCR reactions were incubated at 94, 50, and 72 °C, respectively, for 35 cycles in the thermocycler. The 593 bp and 569 bp PCR products that hybridized to the *L. donovani* and *T. brucei AdoMetDC*s, respectively, were digested with *Bam*HI and *Eco*RI, subcloned into pBluescript KS^+, and sequenced as described above.

Overexpression of L. donovani and T. brucei AdoMetDC Genes in E. coli - The *L. donovani* and *T. brucei AdoMetDC*s were ligated into the pBAce plasmid constructed by Craig et al. (29). The pBAce bacterial expression vector utilizes the bacterial alkaline phosphatase promoter to direct the high level cytoplasmic expression of foreign genes in *E. coli*. To construct the *L. donovani AdoMetDC* expression vector it was necessary to engineer a *Nde*I restriction site at the initiation methionine by PCR. However, because PCR amplification of the entire coding sequence of the *AdoMetDC* of *L. donovani* was not possible the 5' end was amplified, digested with *Nde*I and an internal restriction site, *Sac*I, and spliced together with the *Sac*I-*Sal*I digested 3' end, from the original phage DNA fragment, in a three-way ligation into the *Nde*I/*Sal*I site of the pBAce bacterial expression vector. Thus, introduction of the *Nde*I restriction into the *L. donovani AdoMetDC*site was accomplished by amplification of the 5' end of the *L. donovani AdoMetDC*, using 5'- TCTCATATGAAACACGGTAATTACTCGCTGCGA - 3' as the mutagenic forward primer and 5'-CGCGCTGATTGCCTCGAGGATGTTGGG-3' as the

antisense primer.

With recombinant DNA technology the rapid purification of proteins which are otherwise difficult to isolate can be accomplished by the addition of a hexa- or deca-histidine peptide to the carboxy or amino terminus of a protein. The hexahistidine fusion protein can then be purified over a nickel-agarose affinity column (30). The *L. donovani AdoMetDC* expression construct was therefore further modified to include a carboxy-terminal hexahistidine peptide by PCR amplification of 206 bp of the 3' end of the *L. donovani AdoMetDC* with 5' - GAGAGCCCTGTTGGCAATGCG - 3' containing a *Bgl*I restriction site as the forward primer and 5' - CTCGTCGACATGATGATGATGATGATGGTCGGGCCCACCCTC - 3' containing a *Sal*I restriction site and the complementary sequence encoding six histidines as the antisense primer. The PCR product was digested with the *Bgl*I and *Sal*I restriction enzymes and then ligated into the *Nde*I-*Sal*I digested pBAce expression vector in a three way ligation with a *Nde*I-*Bgl*I restriction digested fragment of the original *AdoMetDC* expression construct described above. The final construct, therefore, contained only the protein coding regions of the *AdoMetDC* gene plus the hexahistidine peptide. The ligation of the *T. brucei AdoMetDC* into pBAce necessitated the introduction of a *Nco*I restriction site at the initiation methionine, because of the presence of two internal *Nde*I restriction sites in the *T. brucei* gene. In a single PCR reaction the entire coding sequence of the *T. brucei AdoMetDC* was amplified with 5'-TCTCCATGCCTCTTGCAAGGACTCTCTTTCG -3' as the *Nco*I containing forward mutagenesis primer and 5'-

TCTGTCGACATGATGATGATGATGTTCCTTCGCTCCAGAAGCTGC -3', encompassing the complimentary sequence for six histidine codons and a *Sal*I restriction site, as the antisense primer.

The respective expression constructs were transformed into the *E. coli* HT551 strain (originally developed by Tabor and Tabor and generously provided by Celia Tabor, Department of Health and Human Services, NIH) (31). This strain contains a deletion in the speED operon and, thus, lacks the gene encoding the bacterial AdoMetDC activity as well as the spermidine synthase gene (31).

500 ml cultures of HT551 bacteria transformed with the recombinant expression plasmid were incubated in a low phosphate medium modified from that previously described (29) by reducing the concentration of "vitamin free" Casamino acids (Difco Laboratories, Detroit, MI) to 0.1 % and deleting the equimolar mixture of NaH_2PO_4 and Na_2HPO_4. The adjustments were crucial for maximizing expression of recombinant protein, as the "vitamin free" Casamino acids contained 100 umol/g of inorganic phosphate. The final concentration of phosphate in the modified induction medium was, therefore, ~100 μM.

Inorganic Phosphate Assay - Inorganic phosphate levels in the *AdoMetDC* expression medium were determined as described (32).

AdoMetDC Assay - AdoMetDC activity was assayed as by the method of Pösö and Pegg with only minor modifications (33). Unless otherwise stated the AdoMetDC activity was assayed in a total volume of 250 μl containing 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 2 mM putrescine, 0.2 mM unlabeled S-adenosylmethionine, and 0.2

μCi of S-adenosyl-L-[carboxy-^{14}C]-methionine and the enzyme preparation, equivalent to 250 - 500 μg of protein. Because the levels of native leishmanial AdoMetDC are approximately 200-fold lower than in trypanosomes, the concentration of unlabeled S-adenosylmethionine was reduced to 0.081 mM in the assay solution. The specific activity of AdoMetDC was determined using the protein concentrations of the enzyme preparations as measured by the methods of Bradford (34).

To characterize the turnover rate of the *T. brucei* AdoMetDC 500 ml of a exponentially growing *in vitro* culture of procyclic (TREU667) parasites were grown to a density of 1 X 10^7 cells/ml and incubated with 500 μg/ml of cycloheximide, a potent inhibitor of protein synthesis which is cytotoxic at this concentration. Aliquots of 50 ml were assayed at 0, 1, 2, 4, 8, and 24 hours for AdoMetDC activity.

To characterize the turnover rate of the *L. donovani* AdoMetDC 1 L of an exponentially growing *in vitro* culture of promastigote parasites was grown to a density of 1 X 10^7 cells/ml and incubated with 500 μg/ml of cycloheximide. Aliquots of 200 ml were assayed at 0, 1, 2, 4, and 6 hours for AdoMetDC activity.

[^{35}S]Methionine Incorporation into the Proteins of T. brucei and L. donovani - To determine whether cycloheximide incubation was effective in eliminating protein synthesis in *T. brucei*, [^{35}S]methionine-cysteine (17 μCi/ml final concentration) was added to two 15 ml aliquots of the above culture. Addition of the radioactive amino acids was delayed for ten minutes after addition of cycloheximide to one of the aliquots. At various time points 1.0-ml aliquots were removed from the no drug control and the cycloheximide-treated sample and added to 1.0 ml of 1 N NaOH. H_2O_2 was added to a

final concentration of 3%, and the cultures were incubated for 10 min at 37°C. Thereafter, 2.0 ml of ice-cold 40% trichloroacetic acid was added to each of the reactions followed by a 15-min incubation on ice. Each trichloroacetic acid precipitate was filtered through a Whatman GF/B filter and washed 4 times with 10 ml of 5% trichloroacetic acid. Radioactivity associated with the filters was quantitated by scintillation counting.

Ammonium Sulfate Precipitation of the Recombinant L. donovani AdoMetDC - The optimal measurement of the effect of putrescine and the polyamines on the activity of the AdoMetDC enzyme necessitated the precipitation of the enzyme by 40% (saturation) ammonium sulfate. The pellet was washed 3 times with the 40% ammonium sulfate solution, redisolved in the putrescine-free assay buffer and dialysed for 1-2 days against the putrescine-free assay buffer.

Polyacrylamide Gel Electrophoresis - Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on 4-20% gradient acrylamide slab gels was performed as described (35).

Antibody Production - Antibodies were prepared by immunizing rabbits with the bacterial inclusion bodies, precipitated by centrifugation at 1000 x g for 10 min in a microcentrifuge. Approximately 100 mg of protein was injected in 700 μl of phosphate buffered saline subcutaneously into the rabbits. Booster injections of 100 mg of protein were repeated at three week intervals. Antibody production was monitored by immunoblotting cell extracts from *L. donovani* DI700 cells.

Immunoblotting - Samples in Laemmli sample loading buffer were incubated at

94°C for 5 min and electrophoresed in SDS gels of either 12% or 15% polyacrylamide. Immunoblotting was performed as described by Towbin et al. (36). The antigen-antibody complex was detected using the ECL Western blotting kit from Amersham Life Science (Buckinghamshire, England), which uses horseradish peroxidase-labelled anti-rabbit goat antibodies as the secondary antibody.

RESULTS

Isolation and Sequencing of the AdoMetDC gene from L. donovani - Preliminary attempts to isolate the *AdoMetDC* from *L. donovani* by cross hybridization under low stringency conditions to the human *AdoMetDC* cDNA (16) were unsuccessful. This is not surprising in light of the low percentage of amino acid sequence conservation between the two enzymes. Consequently, PCR was used to amplify a fragment of the *L. donovani AdoMetDC* from genomic DNA using degenerate oligonucleotide primers complementary to conserved amino acid sequences between the human (16) and yeast protein sequences (17). A 720 bp PCR product that encoded a portion of the *L. donovani AdoMetDC* was amplified and used as a probe for the isolation of the *AdoMetDC* gene from a genomic library. A 5.1 kb *Sal*I fragment was subcloned into pBluescript, and the protein coding segment of the *AdoMetDC* gene was sequenced in both directions. The nucleotide sequence (Fig. 1) contained a single open reading frame of 1,176 nucleotides that encoded a protein of 392 amino acids with a M_r = 44.2 kd for the immature proenzyme and M_r = 33.2 and 11.1 kd for the α- and β-subunits, respectively, of the active enzyme. The active enzyme had a calculated isoelectric point of 4.9. The open reading frame is preceded by an in-frame termination codon (underlined in Fig. 1) beginning at position -30 from the adenylate residue of the predicted initiating methionine codon. PCR of the reverse transcribed RNA indicated that the first nucleotide of the mature *AdoMetDC* transcript (underlined and typed in bold in Fig. 1) is located 146 nucleotides 5' to the start codon, indicating that the methionine at amino acid 10 is not the correct initiation methionine. A physical map of

the 5.1 kb *Sal*I fragment and the sequencing strategy for the *L. donovani AdoMetDC* gene are depicted in Fig. 2.

Isolation and Sequencing of the AdoMetDC gene from T. brucei - Attempts to amplify the *AdoMetDC* from *T. brucei* with similar primers to those used for the leishmanial gene were unsuccessful. Likewise, cross hybridization under low stringency conditions to the leishmanial *AdoMetDC* coding sequence was ineffective. This is a commonly encountered problem with trypanosomatid genes due to the high gaunine-cytosine content, or bias, of the leishmanial genome (19). Consequently, a PCR based strategy was employed to isolate the *T. brucei AdoMetDC* by first amplifying a segment of the *AdoMetDC* gene from a related organism, *T. cruzi*, which is evolutionarily midway between *T. brucei* and *L. donovani* (37) and using the added sequence homology between the two parasite AdoMetDCs to further modify the PCR primer design. Thus, after sequencing a PCR fragment of the *T. cruzi AdoMetDC* amplified from *T. cruzi* genomic DNA using the sequence homology between the two parasite AdoMetDCs and the human and yeast sequences allowed us to determine which amino acids were likely to be conserved in the parasite enzyme and enabled the design of more homologous degenerate oligonucleotide primers. The redesigned primers amplified a 717 bp PCR product encoding a portion of the *T. brucei AdoMetDC* that was used as a probe for the isolation of the *AdoMetDC* gene from a genomic library. A 1.5 kb *Sal*I fragment was subcloned into pBluescript, and the protein coding segment of the *AdoMetDC* gene was sequenced in both directions. The nucleotide sequence (Fig. 3) contained a single open reading frame of 1110 nucleotides that encoded a protein of 370 amino acids with a M_r

= 41.6 kd for the immature proenzyme and a M_r = 31.9 and 9.7 kd for the α- and β-subunits, respectively, of the active enzyme. The active enzyme had a calculated isoelectric point of 4.9. The open reading frame is preceded by an in-frame termination codon (underlined in Fig. 3) beginning at position -42 from the adenylate residue of the predicted initiating methionine codon. PCR of the reverse transcribed RNA indicated that the first nucleotide of the mature *AdoMetDC* transcript (underlined and typed in bold in Fig. 3) is located 143 nucleotides 5' to the start codon. A physical map of the 1.5 kb *Sal*I-*Hinc*II fragment and the sequencing strategy for the *T. brucei AdoMetDC* gene are depicted in Fig. 4.

The predicted amino acid sequences of the *T. brucei* and *L. donovani* AdoMetDC proteins were aligned (Fig. 5) and as expected their primary structures display a high degree of identity, e.g., 63%. The single gap necessary for optimal alignment, between S-187 and D-188 of the trypanosomal protein, was confirmed by sequencing across this region in both directions several times. Further, it is unlikely that a compression of exactly the same three nucleotides would occur in both directions in the *T. brucei* sequence.

Among the aligned human and parasite AdoMetDC sequences (Fig. 6), there are several regions of extensive homology, most notably the proenzyme cleavage site between the E-85 and S-85 of the *T. brucei* AdoMetDC (designated by the vertical lines, ¦, in Fig. 6) and the amino acids necessary for the putrescine stimulation of enzymatic activity and the putrescine stimulation of proenzyme processing (underlined and typed in bold in Fig 6.), as determined by site-directed mutagenesis studies of the human enzyme. In the 12

amino acid region encompassing the proenzyme cleavage site there are 9 completely conserved residues, 2 conservative substitutions, and only one nonconservative substitution.

The predicted amino acid sequences of the *T. brucei* and *L. donovani* AdoMetDC enzymes were also aligned with the primary structures of the yeast (17), potato (38), and *E. coli* (39) enzymes. Both parasite enzymes shared essentially no homology with the *E. coli* enzyme. When each of the AdoMetDCs was examined for amino acid identities in a pairwise fashion to the other eukaryotic enzymes, the *L. donovani* and *T. brucei* AdoMetDCs appeared to be the most divergent (Table I). Despite this lack of overall homology the cleavage site of the *T. brucei* and *L. donovani* proenzymes is identical to the cleavage sites of other eukaryotes, with high conservation of the surrounding eight amino acids in all eukaryotes. The degree of similarity among the eukaryotic AdoMetDCs sequences is, however, generally in good agreement with the observed phylogenetic relationships among the organisms inferred from analysis of their *small subunit rRNA* gene sequences (40), which predicts that the trypanosomes and *Leishmania* are among the earliest organisms to have branched from the eukaryotic lineage.

Structure of the AdoMetDC Gene Locus in L. donovani and T. brucei - Because a number of genes in *L. donovani* and *T. brucei* are arranged in tandemly repeated arrays (41), the *AdoMetDC* locus in the DI700 strain of *L. donovani* and the TREU667 strain of *T. brucei* were analyzed for repeated sequences by restriction enzyme analysis and Southern blotting. *L. donovani* genomic DNA was digested with three restriction enzymes which cut once within the gene and five that did not, transferred to Nytran

filters, and probed with the 1,176 bp *Nde*I-*Sal*I fragment. Restriction enzymes *Sac*I, *Sca*I, and *Bgl*I excised 2 fragments of dissimilar sizes that hybridized to the *AdoMetDC* probe (Fig. 7). If *AdoMetDC* were organized as a tandem repeat within the *L. donovani* genome, this strategy would yield a total of three bands with a single prominent band of identical size in all lanes of a Southern blot in which the genomic DNA had been digested with a single cutter. Moreover, digestion of *L. donovani* DNA with either *Apa*I, *Hind*III, *Sac*II, *Sal*I, or *Kpn*I, which do not cleave within *AdoMetDC*, generates only one DNA fragment that hybridizes to the radiolabeled *AdoMetDC* probe. Although these data suggest that *AdoMetDC* is present as a single copy within the *L. donovani* genome it is possible that several AdoMetDC gene copies do exist on the same chromosome, but are widely separated and not in a tandemly repeated arrangement.

T. brucei genomic DNA was digested with four restriction enzymes which cut once within the gene and four that did not, transferred to Nytran filters, and probed with the 1,110 bp *Nco*I-*Sal*I fragment. Restriction enzymes *Bam*HI, *Spe*I, *Xho*I, and *Sac*I, each of which cleaves the *AdoMetDC* once, excised 2 or more DNA fragments with a single fragment which was a similar size that hybridized to the *AdoMetDC* probe (Fig. 8A). This is consistent with the *T. brucei AdoMetDC* being organized in a complex manner within the *T. brucei* genome, either as multiple copies at several different loci or as a tandem repeat at a single locus or perhaps both. Also supporting this hyothesis is the digest of *T. brucei* genomic DNA with either *Bgl*I or *Cla*I which do not cleave within *AdoMetDC*, generates two DNA fragments that hybridize to the radiolabeled *AdoMetDC* probe.

To further characterize the molecular organization of the *T. brucei AdoMetDC* gene, genomic DNA was digested with limited amounts of *Hind*III enzyme, a restriction enzyme which cuts a single time within the *AdoMetDC* gene, and Southern blotted using the full-length coding sequence of the *T. brucei AdoMetDC* as a probe (Fig. 8B). The restriction pattern in the fifth lane (0.125 Units) suggests that the *AdoMetDC* gene is tandemly repeated at least 4 times with an intergenic region of 1.8 kb, including the coding sequence of the *AdoMetDC* gene. Although this experiment clearly demonstrates that the gene is tandemly repeated at at least one locus, the fact that the intergenic region in Fig. 8A appears to be approximately 10 kb, as suggested by the four digests with enzymes that cut singly within the gene, conflicts with the hypothesis that this is the primary or sole manner in which this gene is represented in the *T. brucei* genome. A more exact characterization of the *AdoMetDC* locus could be obtained by analysis of the genomic locus in a cosmid library if this facet of trypanosomal molecular biology and polyamine metabolism proves to be significant.

Analysis of the T. brucei and L. donovani AdoMetDC Transcripts - Total RNA was isolated from the procyclic forms of the *T. brucei* TRUE667 strain and promastigote forms of the *L. donovani* DI700 strain, separated by formaldehyde denaturing agarose gel electrophoresis, and probed with the 1,110 bp *Nco*I-*Sal*I *T. brucei* fragment and the 1,176 bp *Nde*I-*Sal*I *L. donovani* fragment, respectively, that contained the protein coding portion of the *AdoMetDC*. A single transcript of 1.8 kb for *T. brucei* and 3.1 kb for *L. donovani* was recognized by the respective probes (Fig. 9 and Fig. 10).

Overexpression of AdoMetDC in E. coli - After appropriate PCR mutagenesis, the

L. donovani and *T. brucei AdoMetDC* were ligated into the *E. coli* expression plasmid pBAce and transformed into the HT551 *E. coli* cell line, which lacks AdoMetDC enzymatic activity. To ensure that no mutations had been incorporated during PCR the amplified regions were sequenced verifying that the primary structures of the recombinant and native AdoMetDC enzymes were identical. After inoculation into low phosphate induction medium, the transformed *E. coli* were grown for 12-16 hr. High levels of recombinant *L. donovani* AdoMetDC protein were produced corresponding to between 40 and 60% of the total cellular protein. Unfortunately, the majority of the recombinant protein was present in the insoluble fraction in the form of inclusion bodies. Despite this the small fraction of soluble, active enzyme in bacterial lysates corresponded to between 50 and 100-fold greater levels of AdoMetDC specific activity than in similar lysates of promastigote parasites, e.g. 60 pmoles/m/mg protein as compared to 45 pmoles/h/mg protein (Fig. 11). HT551 E. coli cells transformed with the *T. brucei AdoMetDC* expression construct had much lower levels of AdoMetDC activity, corresponding to between 2% and 5% of the levels detected in procyclic *T. brucei* cell lysates (Fig 12). HT551 cells transformed with a control pBAce plasmid expressed no AdoMetDC activity. Proteins from the induced *E. coli* were fractionated by SDS-PAGE and stained with Coomassie Blue (Fig. 13). In the induced cells the recombinant *L. donovani* AdoMetDC is the predominant protein and despite the fact that most of the protein is insoluble, sufficient amounts of active enzyme were present in lysates to allow the biochemical characterization of the *L. donovani* AdoMetDC enzyme. No recombinant *T. brucei* AdoMetDC could be detected by Coomassie Blue staining

indicating that the low levels of AdoMetDC activity detected probably corresponded to the majority of recombinant protein being produced in the bacteria. It is significant, however, that both the *L. donovani* and *T. brucei* AdoMetDC proenzymes were processed correctly in *E. coli*. This is supported not only by the fact that AdoMetDC activity can be detected in induced cells but also by the fact that the presence of the α- and β-subunits of the recombinant *L. donovani* enzyme can be visualized by Coomassie Blue staining on SDS-PAGE gels of the insoluble fraction.

Kinetic Characterization of L. donovani AdoMetDC - Crude lysates of recombinant AdoMetDC were used to determine the K_m values of the leishmanial enzyme for S-adenosylmethionine. Lineweaver-Burk analysis of the kinetic data indicate a K_m value of 76 μM for the recombinant enzyme (Fig. 14). Due to the low levels of native AdoMetDC activity in *L. donovani* parasite lysates K_m measurements were not possible for the native enzyme, however the K_m for the recombinant enzyme is similar to the reported K_m for the native *T. brucei* AdoMetDC enzyme of 30 μM (11).

Effect of Putrescine and the Polyamines on the L. donovani AdoMetDC - To optimally measure the putrescine stimulation of enzymatic activity it was necessary to free cell extracts of endogenous putrescine by ammonium sulfate precipitation as described by Tekwani et al. (10). Approximately 20% of the recombinant AdoMetDC activity of crude *E. coli* lysates was recovered from the protein fraction precipitated with 40% ammonium sulfate. No AdoMetDC activity was detected in the dialyzed supernatants. The recombinant AdoMetDC exhibited an approximate 3 fold stimulation by putrescine over basal levels with maximal stimulation at about 2 mM. Half-maximal

stimulation was seen at 50 μM concentrations of putrescine (Fig. 15). Micromolar concentrations of the polyamine, spermidine, strongly inhibited the putrescine stimulation of the recombinant AdoMetDC with half-maximal inhibition of the putrescine stimulation at less than 50 μM and returning AdoMetDC activity to basal levels, i.e., the level of enzymatic activity seen without putrescine, at 250 μM spermidine (Fig. 16). 2 mM spermine also inhibited the putrescine stimulation to below basal levels (Fig. 17).

Western Blot Analysis of Native L. donovani Promastigote Cell Lysates - Lysates of *L. donovani* DI700 cells were fractionated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and reacted with rabbit antibodies raised against the insoluble recombinant AdoMetDC enzyme. Western blots of lysates from stationary phase cells revealed two signals with the apparent molecular weights of the α- and β-subunits of the active enzyme. Immunoblots of lysates from exponentially dividing cells revealed a third signal with the apparent molecular weight of the proenzyme, suggesting that the leishmanial AdoMetDC is synthesized as a proenzyme which is later cleaved to obtain the α- and β-subunits. In addition, as the molecular masses of the leishmanial enzyme deduced from the nucleotide sequence and those which are observed from the immunoblot are approximately equivalent, it appears that the leishmanial AdoMetDC is not post translationally modified except for its cleavage to the active α- and β-subunits.

The Enzymatic Half-life of T. brucei and L. donovani AdoMetDC - Exponentially growing procyclic forms of *T. brucei* TREU667 and *L. donovani* DI700 promastigote forms were incubated with [^{35}S]methionine in the presence and absence of 500 μg/ml cycloheximide (Fig. 20A and 21A). The time course of the [^{35}S]methionine incorporation

into the trichloroacetic acid-precipitable fraction of the control cells suggests active protein synthesis during the incubation period. The absence of incorporation into the same fraction of the cycloheximide treated cells indicates that the protein synthesis was effectively inhibited by 500 μg/ml of cycloheximide. During the incubation with cycloheximide the AdoMetDC enzymatic activity in *T. brucei* procyclic forms was assayed and the results indicated that the enzymatic activity of the *T. brucei* AdoMetDC decreased by 1/2 every 3 to 4 hours (Fig 20B). A semi-logarithmic plot of this data indicates that the half-life of the *T. brucei* AdoMetDC enzyme is approximately 3.7 hours. Similarly, the AdoMetDC activity in *L. donovani* promastigotes was measured after inhibition of protein synthesis and the results indicate that the enzymatic half-life of the *L. donovani* AdoMetDC is in excess of 6 hours, the exact duration of which was not measurable under these experimental conditions.

Molecular and Biochemical Characterization of the L. donovani MDL 1000 cell line - Because the actual anti-parasitic mechanism of MDL 73811 remains uncertain (9), we sought to generate a mutant cell line 250-fold less sensitive to growth inhibition by MDL 73811 (Fig. 23). Gene amplification is a common means by which *Leishmania* circumvent drug toxicity as demonstrated by the amplification of the *ODC* gene and an extrachromasomal element in response to DFMO drug pressure (20). Gene amplification of the *AdoMetDC* and the presence of an extrachromasomal element were ruled out by Southern analysis of standard agarose gels (Fig. 24) and pulsed field gels, respectively (Fig. 25). Likewise, Northern analysis of mutant and wild-type total RNA with the *AdoMetDC* coding sequence showed no transcriptional activation (Fig. 26).

Resistance to DFMO in *Leishmania* is purported to occur not only from increased active enzyme but increased inactive enzyme which binds DFMO as well. Thus, to rule out the possibility of translational or post translational activation of the *AdoMetDC,* the AdoMetDC specific activity was measured in mutant and wild-type cells and Western blots of the lysates of exponentially growing promostigotes were performed (Fig 29). Assay of lysates of the *L. donovani* DI700 and MDL 1000 cells showed essentially equivalent AdoMetDC specific activity (Fig. 27), and Western blots showed similarly equal levels of AdoMetDC protein (Fig. 29), thereby ruling out translational activation as a mechanism of drug resistance. The sensitivity of the AdoMetDC enzymes, of the mutant and wild-type cell lines, to inhibition with MDL 73811 were measured over a 1000-fold range of concentrations (Fig. 28) and showed little difference in sensitivity to the drug as well as displaying little overall inhibition. Finally, since MDL 73811 is a purine analog and an alteration in transport activity could theoretically result in resistance to this compound, the patency of the purine transporter was evaluated by assessing the sensitivity of mutant and wild-type cell to toxic purine analogs (Fig. 30). The results indicate that the transport activity of the mutant cell type has not been altered in such a way as to convey a resistant phenotype.

Thus, in light of the experiment showing little AdoMetDC-specific inhibition by MDL 73811 and the fact that the mutant phenotype does not involve any alteration in the molecular biology or biochemistry of the AdoMetDC enzyme, one must conclude that MDL 73811 does not target the leishmanial AdoMetDC. This is somewhat surprising when one considers that the K_i of MDL 73811 for the trypanosomal

AdoMetDC is reported to be 1.5 μM and and suggests that there may be key structural differences between the two enzymes despite their high degree of similarity.

DISCUSSION

The polyamine biosynthetic pathway and the AdoMetDC enzyme represent important targets for the chemotherapy of illnesses as diverse as malignancy and protozoal disease. Here we report the isolation of the full length *AdoMetDC* gene of *T. brucei* and *L. donovani* from their respective genomic libraries, their molecular characterization, functional expression in *E. coli*, and the biochemical characterization of the recombinant *L. donovani* AdoMetDC enzyme.

In summary, the nucleotide sequence disparity of the *T. brucei* and *L. donovani* *AdoMetDC* genes precluded their recognition by the heterologous human *AdoMetDC* probe, and a PCR based strategy was employed to amplify a specific *AdoMetDC* fragment from *L. donovani* gDNA using complementary oligonucleotide primers generated to the conserved amino acid sequences between the human (16) and *S. cerevisiae* (17) AdoMetDC enzymes. Because these primers did not successfully amplify a segment of the *T. brucei AdoMetDC* despite modification to incorporate the homology between the yeast, human, and *L. donovani* sequences, we amplified the corresponding segment of the *T. cruzi AdoMetDC*, and after characterizing the sequence at the forward primer site by using the spliced-leader sequence and PCR, we were able to obtain sufficient new sequence information to design primers which amplified a segment of the *AdoMetDC* from *T. brucei* gDNA. Nucleotide sequence analysis of the isolated genes revealed that the primary structures of the *L. donovani* and *T. brucei* AdoMetDC enzymes exhibited greater similarity to the human and potato AdoMetDCs than to their counterpart from *S. cerevisiae*. The lack of homology between the *T. brucei*, *L.*

donovani, and other eukaryotic AdoMetDCs is not surprising and is consistent with published molecular phylogenic models constructed using *small subunit ribosomal RNA* gene sequences which place *Leishmania* and the trypanosomes among the more primitive eukaryotes (40).

The amino acid sequence divergence among the AdoMetDC proteins from phylogenetically diverse organisms also suggests that conservation of primary structure is not critical for enzyme function, except in certain key regions. From the alignments of these regions among the various AdoMetDC proteins it should be possible to evaluate which amino acid residues are crucial for the maintenance of the structural features required for the catalytic competence of these enzymes and aid in the selection of amino acid residues for site-directed mutagenesis and structure function studies. Of particular interest are the structural differences between the human and parasite enzymes which result in the dissimilar effects of spermidine and spermine on their respective activities.

The ability to over-express the *L. donovani AdoMetDC* in an *E. coli* background genetically deficient in AdoMetDC has allowed us to biochemically characterize the *L. donovani* AdoMetDC enzyme. Our findings indicate that the *L. donovani* AdoMetDC is similar to the human and trypanosomal enzymes (10) in that it is activated by micromolar levels of putrescine, a polyamine biosynthetic intermediate. The leishmanial enzyme is, however, strikingly dissimilar to the human AdoMetDC in that it is strongly inhibited by micromolar levels of spermidine, the product of the pathway, rather than activated, as is the human enzyme (10). This makes good biochemical sense, as the polyamine pathway of higher eukaryotes and mammals produces spermine, for which

spermidine is a biosynthetic intermediate.

The tight control of enzymatic activity by the pathway intermediate and end product, e.g., putrescine and spermidine, is similar to numerous regulatory enzymes in the classic biochemical sense and is indicative of the key role this enzyme plays in the control of the polyamine biosynthetic pathway in trypanosomes and *Leishmania*. This regulatory role, coupled with the dissimilar kinetic characteristics, and greater enzymatic half-lives of the parasite AdoMetDCs in comparison to the human enzyme, make this protein an attractive rational chemotherapeutic target for both of these organisms.

Along these lines, it should be noted that although *L. donovani* and *T. brucei* are relatively closely related evolutionarily, as demonstrated by the 63% identity of their AdoMetDC amino acid sequences, the pathogenesis of their respective diseases is strikingly disparate and the rational design of therapies for their treatment may necessitate fundamentally different approaches. Because *T. brucei* is an extracellular parasite, its differential uptake of purine analogs, e.g., the proposed trypanocidal mechanism of MDL 73811 (42) as compared to human cells, makes inhibitors such as MDL 73811 and related analogs excellent candidates as lead structures for the design of inhibitors of AdoMetDC. By contrast, *Leishmania* reside within the phagocytic vacuole of the host macrophage and are exposed only to compounds which have already crossed the plasma membrane. Although polyamine uptake in *Leishmania* has yet to be extensively characterized it is known that mammalian cells (43) and *Leishmania* (20) both possess polyamine transporters and since the leishmanial AdoMetDC enzymatic activity is strongly inhibited by polyamines such as spermidine, it is possible that

spermidine analogs, such as the experimental antiproliferative drug, bis(ethyl)spermidine, could in the future provide a new rational basis for the design of selective antileishmanial chemotherapies. Along these same lines, Baumann et al. have already demonstrated the efficacy of bis(benzyl)polyamine analogs for the therapy of murine models of leishmaniasis which are purported to function via interference with the normal cellular function of the polyamines (44,45). Whether bis(benzyl)polyamine analogs act in part via inhibition of the AdoMetDC enzyme and whether the combination of bis(benzyl)polyamine analogs with spermidine analogs, which inhibit polyamine biosynthesis through their allosteric effects on AdoMetDC, will increase their parasite specific toxicity is under current investigation.

It is also interesting that *L. donovani* promastigotes exhibit approximately 200-fold less AdoMetDC specific activity than *T. brucei* procyclic cells, e.g., 75 pmol/h/mg protein versus 15 nmol/h/mg protein (10), respectively. Similar levels of *L. donovani* AdoMetDC specific activity, e.g., 57 pmol/h/mg protein, have been measured by Tekwani et. al. (Babu Tekwani, personal communication). This lack of activity has a number of explanations, however, a likely possibility, worthy of further investigation, is that *Leishmania* possess a polyamine transporter by which they salvage host polyamines. The *L. donovani* AdoMetDC specific antibodies will also provide a tool with which to purify and characterize the native enzyme, and possibly shed more light on this interesting biochemical question.

Although virtually all antiparasitic regimens are empirical in design, a precedent for the rationally based therapy of African trypanosomiasis exists, which targets the

polyamine biosynthetic pathway. DFMO, an irreversible inhibitor of ODC, has literally revolutionized the treatment of West African trypanosomiasis (3). The therapeutic efficacy of DFMO is based in part on the rapid rate of turnover of the mammalian enzyme in comparison to the *T. brucei* ODC (46). The instability of the mammalian ODC is conferred by a COOH terminus rich in PEST sequences (47), a sequence missing in the *T. brucei* ODC (46). It has been shown that in some cases, regions of amino acids known as PEST sequences are important for targeting proteins for rapid degradation in mammalian cells (48). From a pharmacologic standpoint it is, therefore, significant that the trypanosomal AdoMetDC turnover rate is significantly greater than that of the human enzyme and this may indeed contribute to the selective anti-trypanosomal action of the experimental enzyme inhibitor MDL 73811, as proposed for DFMO and ODC.

It is also of interest that the leishmanial and trypanosomal enzymes have significantly longer enzymatic half-lives than their mammalian counterpart, in spite of the fact that they both contain carboxyl-terminal regions that are purported to be PEST sequences in the mammalian enzyme (16). One of the longest stretchs of amino acid sequence similarity among the eukaryotic AdoMetDCs occurs towards the COOH terminus in which 17 identical and 9 similar amino acids are found within a 34 amino acid stretch that corresponds to residues 244 and 277 of the *T. brucei* AdoMetDC. Within this region of the human enzyme, from Thr-241 to Thr-257, 10 of 17 amino acids are PEST residues. Of these residues, 8 are conserved between the parasite and human AdoMetDC sequences, e.g., TIHITPEPEFSYVSFET. Although no known function has

been attributed to this region of the protein, it is known that Glu-275 (of the *T. brucei* protein) is necessary for the putrescine activation of processing and stimulation of enzymatic activity in the human enzyme (38). One possible explanation is that it is simply serendipitous that this region contains a high percentage of PEST residues that are structurally important, explaining their conservation in the sequences, and that their presence does not play a role in the short half-life of the human enzyme. Alternatively, they could be important for both the structural integrity as well as the short half-life of the human enzyme and the trypanosomes and *Leishmania* could lack a mechanism by which to target proteins for degradation. This latter hypothesis has been proposed to explain why proteins containing engineered PEST sequences have extended half-lives in trypanosomes, whereas the same proteins are rapidly degraded in mammalian cells (47).

Finally, it should be noted that although DFMO treatment does not manifest the toxic side effects of the conventional antitrypanosomal drugs and is effective for the treatment of West African sleeping sickness after central nervous system involvement, its administration is prolonged and expensive. Furthermore, the drug is not particularly effective toward the agent of the more fulminant East African form of sleeping sickness, *T. b. rhodesiense* (4). Likewise, the chemotherapy of leishmaniasis is far from adequate and has changed little for over 50 years. Thus, the search for newer and improved therapies for the treatment of African trypanosomiasis and leishmaniasis remains an important goal. The isolation of the genes encoding the *T. brucei* and *L. donovani* AdoMetDC enzymes and the functional over-expression of recombinant *L. donovani AdoMetDC* in *E. coli* provide an avenue for a structure-based strategy for the design and

discovery of novel AdoMetDC substrate analogs or AdoMetDC inhibitors that can serve as effective agents in the treatment of African trypanosomiasis, leishmaniasis, and perhaps other parasitic diseases as well.

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REFERENCES

1. Berman JD. Chemotherapy for leishmaniasis: biochemical mechanisms. Reviews of Infectious Diseases 1988; 10:560-586.

2. Grimaldi G, Tesh RB. Leishmaniases of the New World: Current Concepts and Implications for Future Research. Clinical Microbiology Reviews 1994; 6:230-250.

3. Kuzoe FAS. Current situation of African trypanosomiasis. Acta Tropica 1993; 54:153-162.

4. Bacchi CJ, Garofalo J, Ciminelli M, et al. Resistance to DL-alpha-difluoromethylornithine by clinical isolates of *Trypanosoma brucei rhodesiense.* Role of S-adenosylmethionine. Biochemical Pharmacology 1993; 46:471-481.

5. Bacchi CJ, Nathan HC, Livingston T, et al. Differential susceptibility to DL-alpha-difluoromethylornithine in clinical isolates of *Trypanosoma brucei rhodesiense.* Antimicrobial Agents and Chemotherapy 1990 Jun;34(6):1183-1188

6. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Research 1988; 48:759-774.

7. Fairlamb AH. Trypanothione metabolism and rational approaches to drug design.

Biochemical Society Transactions 1990; 18:717-720.

8. Fairlamb AH, Henderson GB, Bacchi CJ, Cerami A. In vivo effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. Molecular and Biochemical Parasitology 1987; 24:185-191.

9. Bitonti AJ, Byers TL, Bush TL, et al. Cure of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* infections in mice with an irreversible inhibitor of S-adenosylmethionine decarboxylase. Antimicrobial Agents and Chemotherapy 1990; 34:1485-1490.

10. Tekwani BL, Bacchi CJ, Pegg AE. Putrescine activated S-adenosylmethionine decarboxylase from *Trypanosoma brucei brucei*. Molecular and Cellular Biochemistry 1992; 117:53-61.

11. Bitonti AJ, Dumont JA, McCann PP. Characterization of *Trypanosoma brucei brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(gaunylhydrazone). Biochemical Journal 1986; 237:685-689.

12. Iovannisci DM, Ullman B. High efficiency plating method for *Leishmania* promastigotes in semi-defined or completely defined medium. Journal of Parasitology 1986; 169:633-636.

13. Brun R, Schonenberger M. Cultivation and in vitro cloning of procyclic culture forms of Trypanosoma brucei in a semi-defined medium. Short communication. Acta Tropica 1979; 36:289-292.

14. Wilson K, Collart FR, Huberman E, Stringer JR, Ullman B. Amplification and molecular cloning of the *IMP dehydrogenase* gene of *Leishmania donovani*. Journal of Biological Chemistry 1991; 266:1665-1671.

15. Landfear SM, Wirth DF. Structures of the mRNA encoded by tubulin genes in *Leishmania enrietti*. Molecular and Biochemical Parasitology 1985; 15:61-82.

16. Pajunen A, Crozat A, Janne OA, et al. Structure and regulation of mammalian S-adenosylmethionine decarboxylase. Journal of Biological Chemistry 1988; 263:17040-17049.

17. Kashiwagi K, Taneja SK, Liu TY, Tabor CW, Tabor H. Spermidine biosynthesis in *Saccharomyces cerevisiae*. Biosynthesis and processing of a proenzyme form of S-adenosylmethionine decarboxylase. Journal of Biological Chemistry 1990; 265:22321-22328.

18. Sanger R, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science USA 1977; 74:5463-5467.

19. Langford CK, Ullman B, Landfear SM. *Leishmania*: codon utilization of nuclear genes. Experimental Parasitology 1992; 74:360-361.

20. Hanson S, Adelman J, Ullman B. Amplification and molecular cloning of the *ornithine decarboxylase* gene of *Leishmania donovani*. Journal of Biological Chemistry 1992; 267:2350-2359.

21. Higgins DG, Sharp PM. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 1988; 73:237-244.

22. Dayhoff MO. Atlas of Protein Sequence and Structure. Washington, D.C.: National Biomedical Research Foundation, 1978:345-358.

23. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor University Press, 1989.

24. Lehrach H, Diamond D, Wosney J, Boedker H. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 1977; 16:4743-4751.

25. Kawasaki ES. Amplification of RNA. In: Innis MA, Gelfand DH, Sninsky JJ, White

TJ, eds. PCR Protocols. A Guide to Methods and Applications. San Diego, CA: Academic Press, 1990:21-27.

26. Wilson K, Hanson S, Landfear S, Ullman B. Nucleotide sequence of the *Leishmania donovani* medRNA gene. Nucleic Acids Research 1991; 19:5787.

27. Murphy WJ, Watkins KP, Agabian N. Identification of a novel Y branch structure as an intermediate in Trypanosome mRNA processing: evidence for trans splicing. Cell 1986; 47:517-525.

28. Sutton RE, Boothroyd JC. Evidence for trans splicing in Trypanosomes. Cell 1986; 47:527-535.

29. Craig SP, Yuan L, Kuntz DA, McKerrow JH, Wang CC. High level expression in Escherichia coli of soluble, enzymatically active shistosomal hypoxanthine/guanine phosphoribosyltransferase and trypanosomal ornithine decarboxylase. Proceedings of the National Academy of Sciences USA 1994; 88:2500-2504.

30. Bush GL, Tassin A, Friden H, Meyer DI. Purification of a translocation-competent secretory protein precursor using Nickel ion affinity chromatography. Journal of Biologic Chemistry 1991; 266:13811-13814.

31. Tabor CW, Tabor H, Xie Q. Spermidine synthase of Escherichia coli: Localization of the spe2 gene. Proceeding of the National Academy of Sciences USA 1986; 83:6040-6044.

32. Ames BN, Dubin DT. . Journal of Biological Chemistry 1960; 235:769-775.

33. Pegg AE, Poesoe H. S-adenosylmethionine decarboxylase (rat liver). Methods Enzymol 1983; 94:234-239.

34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976; 72:248-254.

35. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-685.

36. Towbin H, Staehelin T, Gordon J. Electrophorectic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences USA 1979; 76:4350-4354.

37. Lake JA, Vidal F, Paulo CG, Carlos M, Simpson L. Evolution of parasitism: Kinetoplastid protozoan history reconstructed from mitochondrial rRNA gene sequences.

Proceedings of National Academy of Sciences USA 1988; 85:4779-4783.

38. Stanley BA, Shantz LM, Pegg AE. Expression of Mammalian S-Adenosylmethionine Decarboxylase in *Escherichia coli*: determination of sites for putrescine activation of activity and processing. Journal of Biological Chemistry 1994; 269:11:7901-7907.

39. Tabor CW, Tabor H. The speEseD Operon of *Escherichia coli*: Formation and processing of a proenzyme form of S-adenosylmethionine decarboxylase. Journal of Biological Chemistry 1987; 262:16037-16040.

40. Sogin ML, Elwood HJ, Gunderson JH. Evolutionary diversity of eukaryotic small-subunit rRNA genes. Proceeding of the National Academy of Sciences USA 1986; 83:1383-1387.

41. Langford CK, Ewbank SA, Hanson SS, Ullman B, Landfear SM. Molecular characterization of two genes encoding members of the glucose transporter superfamily in the parasitic protozoan *Leishmania donovani*. Molecular and Biochemical Parasitology 1992; 55:51-64.

42. Byers TL, Casara P, Bitonti AJ. Uptake of the antitrypanosomal drug 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine (MDL 73811) by the purine transport system of *Trypanosoma brucei brucei*. Biochemical Journal 1992; 283:755-758.

43. Pegg AE, McCann PP. Polyamine metabolism and function. American Journal of Physiology 1982; 243:C212-C221.

44. Baumann RJ, Hanson WL, McCann PP, Sjoerdsma A, Bitonti AJ. Suppression of both antimony-susceptible and antimony-resistant *Leishmania donovani* by abis(benzyl)polyamine analog. Antimicrobial Agents and Chemotherapy 1990; 34:722-727.

45. Baumann RJ, McCann PP, Bitonti AJ. Suppression of *Leishmania donovani* by oral administration of a bis(benzyl)polyamine analog. Antimicrobial Agents and Chemotherapy 1991; 35:1403-1407.

46. Phillips MA, Coffino P, Wang CC. Cloning and sequencing of the *ornithine decarboxylase* gene from *Trypanosoma brucei*. The Journal of Biological Chemistry 1987; 262:8721-8727.

47. Ghoda L, Van Daalen Wetters T, Macrae M, Asherman D, Coffino P. Prevention of rapid intracellular degradation of ODC by a carboxy-terminal truncation. Science 1989; 243:1493-1495.

48. Rogers S, Wells R, Rechsteiner M. Amino Acid Sequences Common to Rapidly Degraded Proteins: The PEST Hypothesis. Science 1986; 234:364-369.

FIGURES

FIG. 1. **Nucleotide and predicted amino acid sequence of the protein coding region of the *L. donovani AdoMetDC*.** 1,451 bp of the 5.1 kb *Sal*I-*Kpn*I fragment were sequenced in both directions and the amino acid sequence inferred from its 1,176 bp open reading frame. Nucleotides within the protein coding region of the *AdoMetDC* are enumerated positively from the first adenylate nucleotide of the predicted initiating Met, and amino acids within the AdoMetDC protein are numbered positively from the first Met. Nucleotides within the 5' untranslated region are enumerated negatively from the same adenylate moiety. An in-frame termination codon 5' to the predicted start site is underlined and the nucleotide that corresponds to the first nucleotide of the mature *AdoMetDC* transcript to which the mini-exon is *trans*-spliced is typed in bold and underlined.

Nucleotide and Predicted Amino Acid Sequence of the *L. donovani AdoMetDC*

```
-165 TTTGGCCGAG GTGCTTCAGC GTACCGAAAA ATCTGTGCTT CATCGCCTCT TGCTTTTCCC CCACCCCAAC AGATCTGCGG CGGTCGCCAC   -76
 -75 TCCTTTCCAC AGATTCGTGC ACTTTCTCTG ATACACCCAG GCGCGTAAAG AGCGGCTCTG AGGCGGCGCA CACAC                    -1

   1 ATG AAA CAC GGT AAT TAC TCG CTG GCA ACC ATG AAT GTC TGC TCG AAC ACC ACA AAG GAC CCC CTC ACG CTC ATG    75
     Met Lys His Gly Asn Tyr Ser Leu Ala Thr Met Asn Val Cys Ser Asn Thr Thr Lys Asp Pro Leu Thr Leu Met
     1

     GCG ATG TGG GGC TCC ATG AAA GGG TAC AAC CCG GAG CAA GGG TTC AGC TTC GAA GGT CCG GAC AAG CGC CTC GAG   150
     Ala Met Trp Gly Ser Met Lys Gly Tyr Asn Pro Glu Gln Gly Phe Ser Phe Glu Gly Pro Asp Lys Arg Leu Glu
     26

     GTG ATT TTG CGC TGT ACA CTG GAG ACG CAC CTG GAT GGG CTG CGC AGC CTC GAC GAC TCC GTG TGG TCC GGT GTG   225
     Val Ile Leu Arg Cys Thr Leu Glu Thr His Leu Asp Gly Leu Arg Ser Leu Asp Asp Ser Val Trp Ser Gly Val
     51

     GTT GGC TGC CTC AAC GCG CAG ATC GTG TCG AGG GAG TCG AAC GAG TAC ATA AAC AGC TAC GTT CTG AAC GAG AGC   300
     Val Gly Cys Leu Asn Ala Gln Ile Val Ser Arg Glu Ser Asn Glu Tyr Ile Asn Ser Tyr Val Leu Asn Glu Ser
     76

     TCT CTC TTT GTG ATG AAA AAC CGC ATT ATT CTT ATC ACA TGC GGC ACC ACC ACA TTG CTT AAC AGC ATC CCC AAC   375
     Ser Leu Phe Val Met Lys Asn Arg Ile Ile Leu Ile Thr Cys Gly Thr Thr Thr Leu Leu Asn Ser Ile Pro Asn
     101

     ATC CTC GAG GCA ATC AGC GCG GTG CGT GGG GAG CTG GAG TGG GTG TCC TTC ATG CAC AAA AAC TAC TCC TTT CCG   450
     Ile Leu Glu Ala Ile Ser Ala Val Arg Gly Glu Leu Glu Trp Val Ser Phe Met His Lys Asn Tyr Ser Phe Pro
     126

     TGG ATG CAG AAA GGG CCG CAC ACG TCC CTG GCG GAC GAG TTT GCG ACA CTG AAG CAG CAC TTC CCT ACT GGC AAG   525
     Trp Met Gln Lys Gly Pro His Thr Ser Leu Ala Asp Glu Phe Ala Thr Leu Lys Gln His Phe Pro Thr Gly Lys
     151

     CCT TAC ATC TTC GGC CCT GTG GAC AGC GAC CAT TAC TTT CTC TTC TGT TAC GAT GAC ATC ATC CGC CCC TGC AGC   600
     Pro Tyr Ile Phe Gly Pro Val Asp Ser Asp His Tyr Phe Leu Phe Cys Tyr Asp Asp Ile Ile Arg Pro Cys Ser
     176

     TCT GAG GAT GAC ACA CAG CTC AGT ATG ACC ATG TAC GGG CTG GAC AAG GAG CAG ACC AAG CAC TGG TTC AGC GAC   675
     Ser Glu Asp Asp Thr Gln Leu Ser Met Thr Met Tyr Gly Leu Asp Lys Glu Gln Thr Lys His Trp Phe Ser Asp
     201

     CGC TTC ATC TCC ACC AGC GCG GAG ACG GCG GCG ATC CGC GCT GCG ACG CAC CTG GAT CGC GTT GTG GAC GGC ACG   750
     Arg Phe Ile Ser Thr Ser Ala Glu Thr Ala Ala Ile Arg Ala Ala Thr His Leu Asp Arg Val Val Asp Gly Thr
     226

     TGG ACG CTA CAC GAC CTC CAG TTC GAG CCC TGC GGC TAC AGC ATT AAC GCC ATC CGT GAC GAG GAG TAC CAG ACG   825
     Trp Thr Leu His Asp Leu Gln Phe Glu Pro Cys Gly Tyr Ser Ile Asn Ala Ile Arg Asp Glu Glu Tyr Gln Thr
     251

     ATG CAC ATT ACC CCA GAG GAT CAC TGC TCC TTT GCC TCT TAC GAA ACG AAC AGC CGA GCG GCG AAC TAC TCG GAC   900
     Met His Ile Thr Pro Glu Asp His Cys Ser Phe Ala Ser Tyr Glu Thr Asn Ser Arg Ala Ala Asn Tyr Ser Asp
     276

     CGG ATG AAG AAG GTG CTC GGC GTA TTC CGG CCG CAG CGG TTC ACC GTC ATT GTT TTC CTC GAC CCC GAG AGC CCT   975
     Arg Met Lys Lys Val Leu Gly Val Phe Arg Pro Gln Arg Phe Thr Val Ile Val Phe Leu Asp Pro Glu Ser Pro
     301

     GTT GGC AAT GCG TAC AAC GAA GGC AAG GGG ATC GGA GTG GAA CCG GAG TAC TAC CCG GAG TAC AAT CTC CTC CAC  1050
     Val Gly Asn Ala Tyr Asn Glu Gly Lys Gly Ile Gly Val Glu Pro Glu Tyr Tyr Pro Glu Tyr Asn Leu Leu His
     326

     CGC ACC ACG AAC GAG TTC GCG CCG GGC TAC GTG GCT ATG AAG ATC AAC TAC GTC AGG ACG GCT GCG GTG GAA GAG  1125
     Arg Thr Thr Asn Glu Phe Ala Pro Gly Tyr Val Ala Met Lys Ile Asn Tyr Val Arg Thr Ala Ala Val Glu Glu
     351

     ACA GAT ACG GCA GTC GGA GGC GCG GAA CCG GGC GCC GAG GGT GGG CCC GAC TAG                               1179
     Thr Asp Thr Ala Val Gly Gly Ala Glu Pro Gly Ala Glu Gly Gly Pro Asp STP
     376                                                                 392

1178 TCTTGCGCAA AGGTCATCAC CGTGTGCTCG AGACGCTGCA CGCCCACCAA TACATGCAAG CGAGAGAACA AGCAAGGAAA AACTCTGCGC  1269
1270 CTCGCTATTT AATAGGT                                                                                1286
```

FIG. 2. **Physical map and sequencing strategy for the 5.1 kb *Sal*I fragment encompassing the *L. donovani AdoMetDC*.** A partial restriction map of the λLSD3 λGEM-11 clone was obtained by Southern analyses of single and double restriction enzyme digests with enzymes that cut once within the protein coding region of the gene. The solid box indicates the protein coding region of the *AdoMetDC* gene. The direction of transcription is from left to right in the figure. The portions of the gene that encode the α- and β-subunits are indicated. Arrows indicate the segments of DNA for which primers were obtained and the direction in which they were sequenced. Restriction sites are designated at the appropriate points.

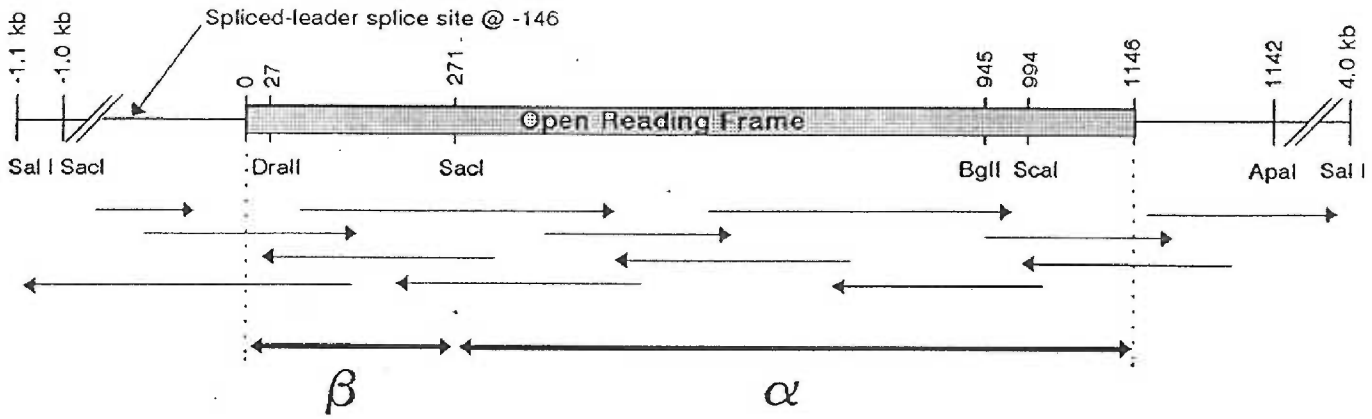

-1.1 kb
-1.0 kb
Spliced-leader splice site @ -146
0
27
271
945
994
1146
1142
4.0 kb
Open Reading Frame
Sal I SacI
DraII
SacI
BglI ScaI
ApaI Sal I
β
α

FIG. 3. **Nucleotide and predicted amino acid sequence of the protein coding region of the *T. brucei AdoMetDC*.** 1,537 bp of the 1.5 kb *Sal*I-*Hinc*II fragment were sequenced in both directions and the amino acid sequence inferred from its 1,110 bp open reading frame. Nucleotides within the protein coding region of the *AdoMetDC* are enumerated positively from the first adenylate nucleotide of the predicted initiating Met, and amino acids within the AdoMetDC protein are numbered positively from the first Met. Nucleotides within the 5' untranslated region are enumerated negatively from the same adenylate moiety. An in-frame termination codon 5' to the predicted start site is underlined and the nucleotide that corresponds to the first nucleotide of the mature *AdoMetDC* transcript to which the mini-exon is *trans*-spliced is typed in bold and underlined.

Nucleotide and Predicted Amino Acid Sequence of the *T. brucei AdoMetDC*

```
-295 GTCGACTCGC ACTTGTGATG TAGGCACCAC TAATACCGTC CCTTTCCCTT CTCTTTCTTT TCTCTTCTCC CTATTCTATC TTATTCATAT  -206
-205 TTTATTTCCC TCGTTTCGGA TTTTTGACCT TTTTCTGTGG AGTTTGTACG CGTATCCTCG AGCACAAAAA GTACATTGTT TGTTGAGCTT  -116
-115 GCACTCTTAG GAAGCGCACC ATTCCTTTGC GGGCCTGCGT GTGCTGCGCT GTCTGGTTTG GTTATTTAAT CCGTAAGGTG CTATATTTGA   -26
 -25 CCGCACACTG ACTGAAGTAC TAGTC                                                                          -1

   1 ATG TCC TCT TGC AAG GAC TCT CTT TCG CTC ATG GCG ATG TGG GGT TCC ATC GCT CGT TTT GAC CCA AAG CAC GAG   75
     MET Ser Ser Cys Lys Asp Ser Leu Ser Leu Met Ala Met Trp Gly Ser Ile Ala Arg Phe Asp Pro Lys His Glu
     1

     CGA AGC TTC GAA GGG CCT GAG AAG CGC TTG GAG GTG ATA ATG CGG GTG GTG GAC GGG ACG CAC GTT AGT GGT TTA  150
     Arg Ser Phe Glu Gly Pro Glu Lys Arg Leu Glu Val Ile Met Arg Val Val Asp Gly Thr His Val Ser Gly Leu
     26

     CTT GCA CAC GAT GAC GAT GTG TGG CAA AAA GTT ATT GAT GCT ATA TGT GCA CAC ATT GTT TCT CGT GAG TTT AAT  225
     Leu Ala His Asp Asp Asp Val Trp Gln Lys Val Ile Asp Ala Ile Cys Ala His Ile Val Ser Arg Glu Phe Asn
     51

     GAA TAC ATT CGC TCC TAC GTG TTA TCG GAG AGC TCA TTG TTT GTA ATG AAA GAT CGC GTG ATT CTC ATA ACT TGT  300
     Glu Tyr Ile Arg Ser Tyr Val Leu Ser Glu Ser Ser Leu Phe Val Met Lys Asp Arg Val Ile Leu Ile Thr Cys
     76

     GGT ACC ATC ACT CTT TTG AAT TGC GTG CCG CTC ATA TGC GAG GCG GTG AGT ACT GTT TGC GGC GAA GTA GAA TGG  375
     Gly Thr Ile Thr Leu Leu Asn Cys Val Pro Leu Ile Cys Glu Ala Val Ser Thr Val Cys Gly Glu Val Glu Trp
     101

     GTT TCC TTC ATG CAC AAA AAC TAC AGT TTC CCG TGG GAG CAG AAG GGA CCG CAT CTT TCG ATG GCG GAA GAA TTT  450
     Val Ser Phe Met His Lys Asn Tyr Ser Phe Pro Trp Glu Gln Lys Gly Pro His Leu Ser Met Ala Glu Glu Phe
     126

     AAG ACA CTT AGG TCC CAT TTT CCG TCG GGA CAA CCA TTC ATA TTT GGT CCG ATT GAC AGC GAC CAC TAC TTC TTG  525
     Lys Thr Leu Arg Ser His Phe Pro Ser Gly Gln Pro Phe Ile Phe Gly Pro Ile Asp Ser Asp His Tyr Phe Leu
     151

     TAT TTG GAC AGT GAT GTT GTT CAA CCC AGC TGC AGT GAC GAT GCA CAA CTC AGC ATG ACC ATG TAT GGG CTT GAT  600
     Tyr Leu Asp Ser Asp Val Val Gln Pro Ser Cys Ser Asp Asp Ala Gln Leu Ser Met Thr Met Tyr Gly Leu Asp
     176

     CGC AAT CAA ACG AAA CAC TGG TAC TCG GAT AAG ATG TTG CCT ACG GGT CCT GAA ACC GCA GTG ATA CGG GAG GCA  675
     Arg Asn Gln Thr Lys His Trp Tyr Ser Asp Lys Met Leu Pro Thr Gly Pro Glu Thr Ala Val Ile Arg Glu Ala
     201

     ACG GGG CTC AGT GAA GTT GTA GAT GAT TCA TGG ATC CTT CAC GAC CTT CAG TAT GAG CCA TGT GGC TAC AGC ATA  750
     Thr Gly Leu Ser Glu Val Val Asp Asp Ser Trp Ile Leu His Asp Leu Gln Tyr Glu Pro Cys Gly Tyr Ser Ile
     226

     AAT GCG ATA CGT GGT AGC GAG TAT CAA ACG ATA CAC ATA ACA CCT GAG GAG CAC TGT TCG TTT GCA TCA TAT GAG  825
     Asn Ala Ile Arg Gly Ser Glu Tyr Gln Thr Ile His Ile Thr Pro Glu Glu His Cys Ser Phe Ala Ser Tyr Glu
     251

     ACG AAT ACG TGT GCT CTT AAT TAC TCG AAG TGT ATC TGT GGA GTA TTG AGA GTG TTC GAC CCT GAA CGG TTT TCT  900
     Thr Asn Thr Cys Ala Leu Asn Tyr Ser Lys Cys Ile Cys Gly Val Leu Arg Val Phe Asp Pro Glu Arg Phe Ser
     276

     GTG ATT GTA TTT ATT GAC CCT GAT AGT GCT GTG GGG AAG TCT TAC CAT AGT GGG GGA ACG ATT GGT GTG GAA CCC  975
     Val Ile Val Phe Ile Asp Pro Asp Ser Ala Val Gly Lys Ser Tyr His Ser Gly Gly Thr Ile Gly Val Glu Pro
     301

     GAG TAT TAT CCG AAC TAT GAA GCG CAT CAC CGT ACA GTG AAC GAG TAT ACA CCG GGT CAC TGG GTT CTG AAG GTA 1050
     Glu Tyr Tyr Pro Asn Tyr Glu Ala His His Arg Thr Val Asn Glu Tyr Thr Pro Gly His Trp Val Leu Lys Val
     326

     AAC TAT GTA AAA CGT GCG GTT GGC ACT GTA GGG ACT TCC GCA GCT TCT GGA GCG AAG GAA TGA                1113
     Asn Tyr Val Lys Arg Ala Val Gly Thr Val Gly Thr Ser Ala Ala Ser Gly Ala Lys Glu Stp
     351                                                                          370

1114 ACGGAGCAGC CCTTGTGCAT TTTGGTTGGT TTCTTGTCTT CGAAGTAGTC TTCATATGCG CAGGCAGATT TTCCCCCTGC TAATGTCTTT  1203
1204 TTCATTCGCC ATCTAGCTGC TATCAGTCGG TTTAAGTTA                                                          1242
```

FIG. 4. **Physical map and sequencing strategy for the 1.46 kb *Sal*I-*Hinc*II fragment encompassing the *T. brucei AdoMetDC*.** A partial restriction map of the λTSD1 EMBL3 clone was obtained by Southern analyses of single and double restriction enzyme digests with enzymes that cut once within the protein coding region of the gene. The solid box indicates the protein coding region of the *AdoMetDC* gene. The direction of transcription is from left to right in the figure. The portions of the gene that encode the α- and β-subunits are indicated. Arrows indicate the segments of DNA for which primers were obtained and the direction in which they were sequenced. Restriction sites are designated at the appropriate points.

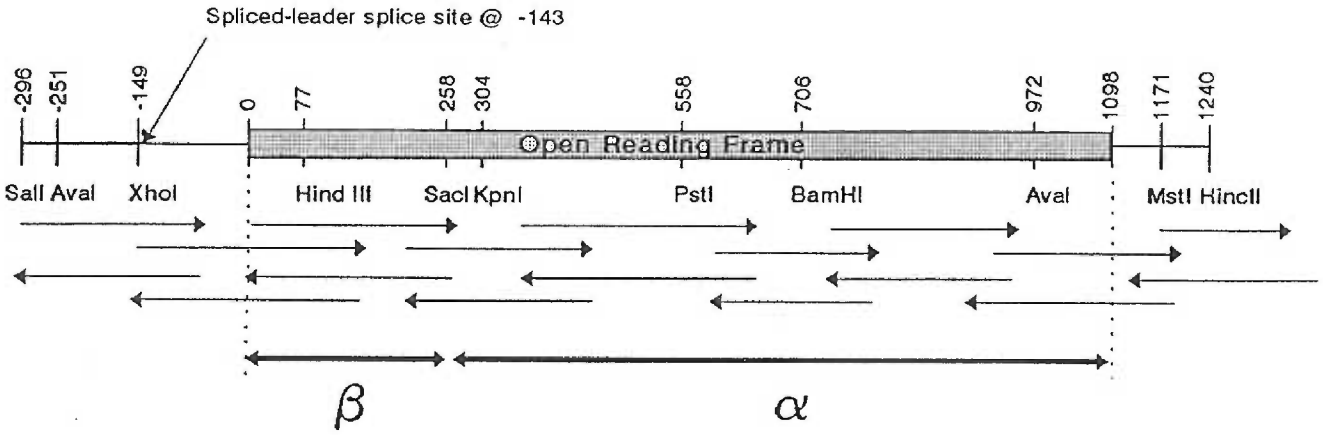

Spliced-leader splice site @ -143
-296
-251
-149
0
77
258
304
558
706
972
1098
1171
1240
Open Reading Frame
SalI AvaI
XhoI
Hind III
SacI KpnI
PstI
BamHI
AvaI
MstI HincII
β
α

FIG. 5. **Alignment of the amino acid sequences of the *T. brucei* and *L. donovani* AdoMetDCs.** The predicted primary structures of the *T. brucei* and *L. donovani* AdoMetDC proteins were aligned according to the CLUSTAL V program, a modification of that described by Higgins and Sharp (21). Amino acids identical between the aligned AdoMetDC sequences are indicated by vertical lines between the aligned sequences, whereas nonidentical amino acids with similarity scores >10, as calculated by the log-odds amino acid similarity matrix of Dayhoff (22), are denoted with a dot between the amino acid sequences. The oligonucleotide primers used to amplify the respective PCR products from gDNA are underlined and typed in bold. The nested PCR primer which was used to identify the correct PCR fragment is typed in bold. Amino acid positions of the two proteins are designated numerically on the right.

Alignment of the T. brucei and L. donovani AdoMetDC protein sequences

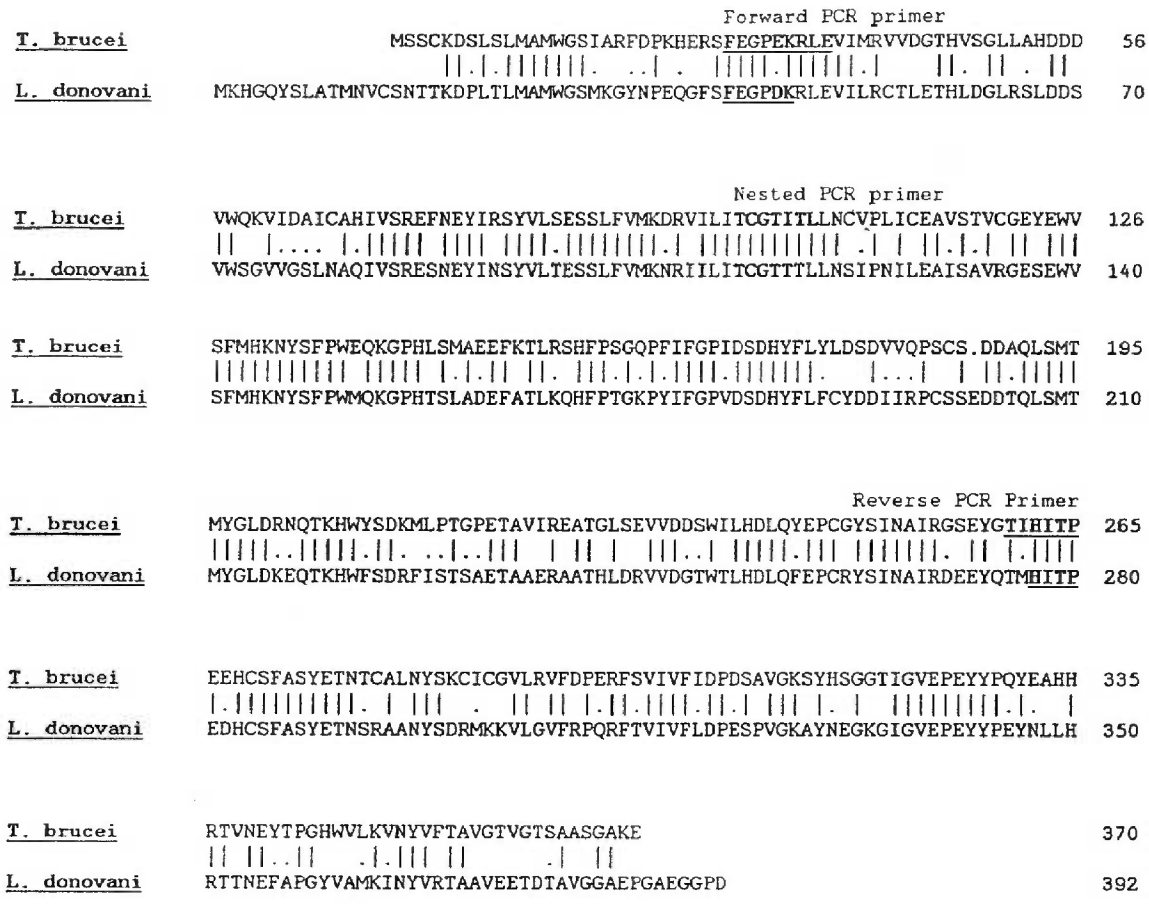


FIG. 6. **Alignment of the amino acid sequences of the *T. brucei, L. donovani*, and human AdoMetDCs.** The predicted primary structure of the *T. brucei AdoMetDC* protein was aligned with those deduced from the nucleotide sequences of the *L. donovani AdoMetDC* and the human *AdoMetDC* cDNA (16) according to the CLUSTAL V program as described in figure 3. Amino acids identical among the three aligned AdoMetDC sequences are indicated by asterisks below the aligned sequences, whereas nonidentical amino acids with similarity scores >10 are denoted with a dot below the amino acid sequences. Amino acid positions of the three proteins are designated numerically on the right. The amino acid residues that have been determined to be important for the putrescine stimulation of processing (38) are designated in bold and underlined.

Alignment of the *T. brucei*, *L. donovani*, and Human AdoMetDC protein sequences

```
T. brucei       ----------MSSCKDSLSLMAMWGSIARFDPKHERSFEGPEKRLE   36
L. donovani     MKHGQYSLATMNVCSNTTKDPLTLMAMWGSMKGYNPEQGFSFEGPDKRLE   50
Human           ----------------------------------MEAAHFFEGTEKLLE   15
                                                   *** .* **

T. brucei       VIMRVVDGTHVSGLLAHDDDV---WQKVIDAICAHIVSREFNEYIRSYVL   83
L. donovani     VILRCTLETHLDGLRSLDDSV---WSGVVGSLNAQIVSRESNEYINSYVL   97
Human           VWFSRWWPDANQGSGDLPTIPRSEWDILLKDVQCSIISVTKTDKQEAYVL   65
                *          *          *  ..  .  *.*.  .*  ***
                 ||
T. brucei       SESSLFVMKDRVILITCGTITLLNCVPLICEAVSTVCG--EYEWVSFMHK  131
L. donovani     TESSLFVMKNRIILITCGTTTLLNSIPNILEAISAVRG--ESEWVSFMHK  145
Human           SESSMFVSKRRFILKTCGTTLLLKALVPLLKLARDYSGFDSIQSFFYSRK  115
                .***.** * * ** ****  **. .  .        *   .  . .*

T. brucei       NYSFPWEQKGPHLSMAEEFKTLRSHFPSGQPFIFGPIDSDHYFLYLDSDV  181
L. donovani     NYSFPWMQKGPHTSLADEFATLKQHFPTGKPYIFGPVDSDHYFLFCYDDI  195
Human           NFMKPSHQGYPHRNFQEEIEFLNAIFPNGAAYCMGRMNSDCWYLYTLDFP  165
                *.  *  *  **  .  .*.  *   ** *  .  * ..**  .*.

T. brucei       VQPSCS-DDAQLSMTMYGLDRNQTKHWYSDKMLPTGPETAVIREATGLSE  230
L. donovani     IRPCSSEDDTQLSMTMYGLDKEQTKHWFSDRFISTSAETAAERAATHLDR  245
Human           ESRVISQPDQTLEILMSELDPAVMDQFY---MKDGVTAKDVTRESGIRD  211
                     *  *  * . *  **      .  .    .  .  .      .  .

T. brucei       VVDDSWILHDLQYEPCGYSINAIR-GSEYGTIHITPEEHCSFASYETNTC  279
L. donovani     VVDGTWTLHDLQFEPCRYSINAIR-DEEYQTMHITPEDHCSFASYETNSR  294
Human           LIPGS-VIDATMFNPCGYSMNGMKSDGTYWTIHITPEPEFSYVSFETNLS  260
                .. ..  ..   ..** **.*... .  * *.***** . *. *.***

T. brucei       ALNYSKCICGVLRVFDPERFSVIVFIDPDS---AVGKSYHS---------  317
L. donovani     AANYSDRMKKVLGVFRPQRFTVIVFLDPES---PVGKAYNE---------  332
Human           QTSYDDLIRKVVEVFKPGKFVTTLFVNQSSKCRTVLRSPQKIEGFK----  306
                  .*   .  *. ** * **   .*.  *   * .

T. brucei       GGTIGVEPEYYPQYEAHHRTVNEYTPGHWVLKVNYVFTAVGTVGTSAASG  367
L. donovani     GKGIGVEPEYYPEYNLLHRTTNEFAPGYVAMKINYVRTAAVEETDTAVGG  382
Human           --RLDCQSAMFNDYN------------------FVFTSFAKKQQQQQS   334
                   .. ..  . .*.                  .* *.

T. brucei       AKE                                               370
L. donovani     AEPGAEGGPD                                        392
Human
```

FIG. 7. **Structure of the *L. donovani AdoMetDC* gene locus.** 10 μg-aliquots of genomic DNA isolated from *L. donovani*, strain DI700, was incubated overnight with the indicated restriction enzymes, fractionated on a 0.8 % agarose gel, transferred to a Nytran filter, and probed with the *Nde*I-*Sal*I DNA segment of the pBAce expression vector. The protein coding region of the *L. donovani AdoMetDC* contains a single restriction site each for *Sac*I, *Bgl*I, and *Pst*I, while *Sac*II, *Sal*I, *Hind*III, and *Kpn*I do not cleave within the *AdoMetDC* coding region. DNA markers are products of λ phage DNA digested with *Ava*I.

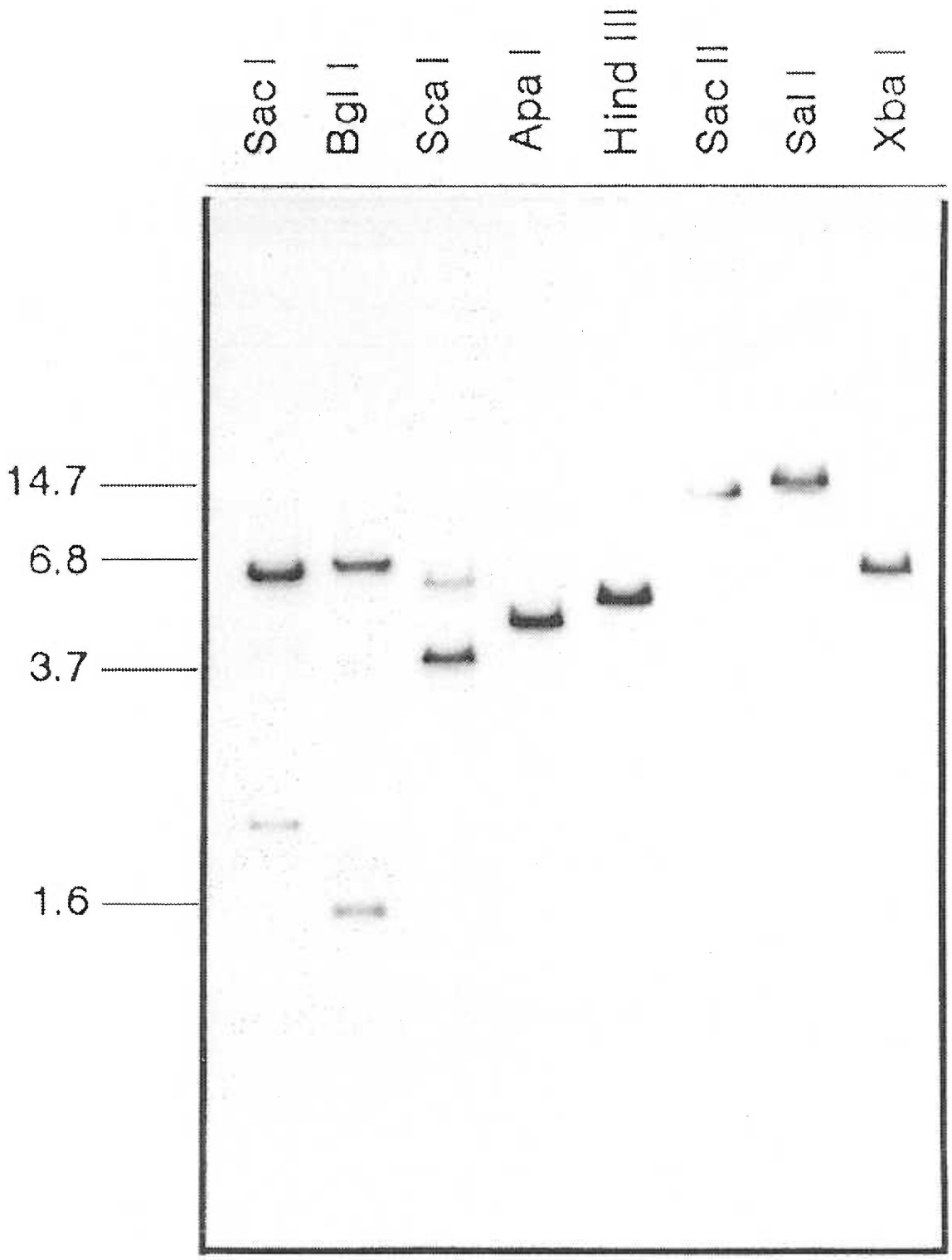
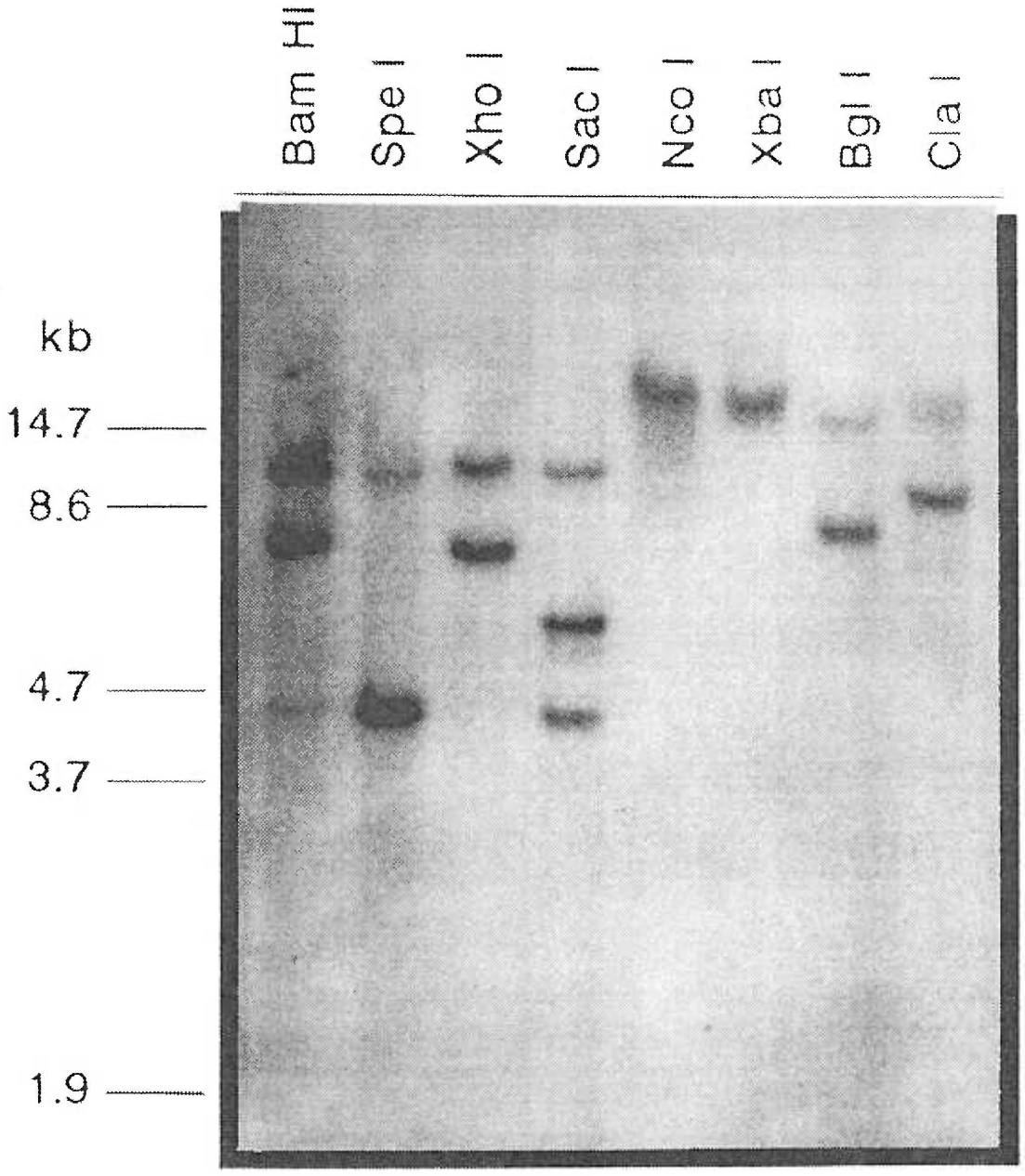

Sac I
Bgl I
Sca I
Apa I
Hind III
Sac II
Sal I
Xba I
14.7
6.8
3.7
1.6

FIG. 8. **Structure of the *T. brucei AdoMetDC* gene locus.** A. 10 μg-aliquots of genomic DNA isolated from *T. brucei*, strain TREU667, were incubated overnight with the indicated restriction enzymes, fractionated on a 0.8 % agarose gel, transferred to a Nytran filter, and probed with the 1,110 bp *Nco*I-*Sal*I *AdoMetDC* fragment. The protein coding region of the *T. brucei AdoMetDC* contains a single restriction site each for *Bam*HI, *Spe*I, *Xho*I, and *Sac*I, while *Nco*I, *Xba*I, *Bgl*I, and *Cla*I do not cleave within the *AdoMetDC* coding region. DNA markers are products of λ phage DNA digested with *Ava*I and were obtained from United States Biochemical (Cleveland, OH). B. 10 μg-aliquots of genomic DNA isolated from *T. brucei*, strain TREU667, were incubated overnight with varying quantities of the *Hind*III restriction enzyme, fractionated on a 0.8 % agarose gel, transferred to a Nytran filter, and probed with the 1,110 bp *Nco*I-*Sal*I *AdoMetDC* fragment. The protein coding region of the *T. brucei AdoMetDC* contains a single restriction site for *Hind*III.


Bam HI
Spe I
Xho I
Sac I
Nco I
Xba I
Bgl I
Cla I
kb
14.7
8.6
4.7
3.7
1.9

Limit Digest of *T. b. brucei* Genomic DNA

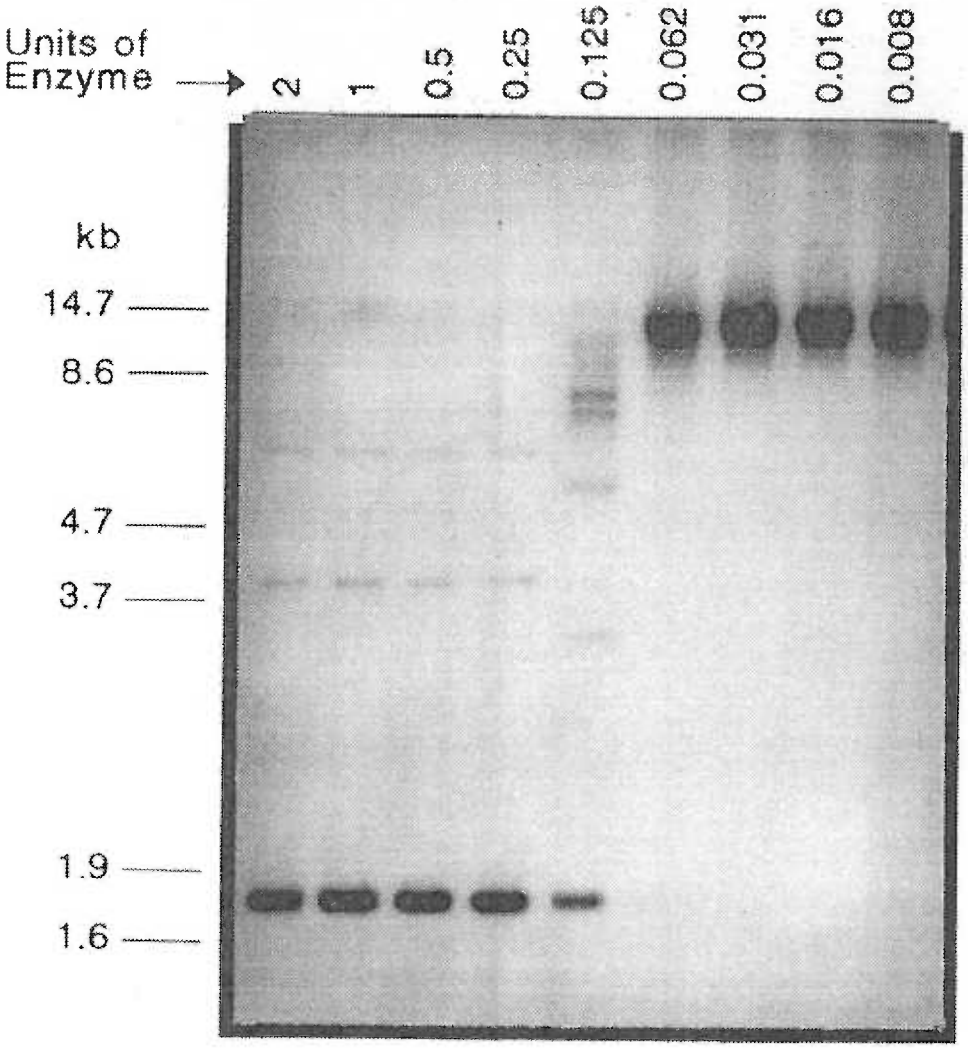


FIG. 9. **Northern blot analysis of the *L. donovani AdoMetDC* transcript.** Total RNA isolated from exponentially growing procyclic forms of *L. donovani*, DI700 strain, was separated by denaturing gel electrophoresis, transferred to a nylon membrane, and probed with the protein coding region of the 1,176 bp *L. donovani Nde*I-*Sal*I *AdoMetDC* fragment from the pBAce expression vector under high stringency conditions. The RNA standards are from the 1.4 - 9.5 kb RNA ladder supplied by GIBCO BRL (Gaithersburg, MD).

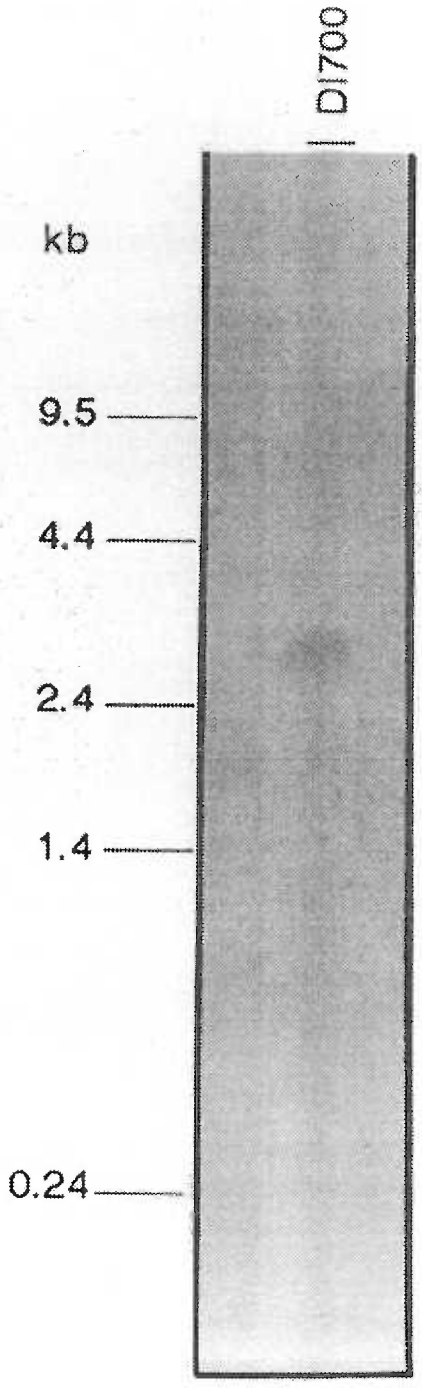

DI700
kb
9.5
4.4
2.4
1.4
0.24

FIG. 10. **Northern blot analysis of the *T. brucei AdoMetDC* transcript.** Total RNA isolated from exponentially growing procyclic forms of *T. brucei*, strain TREU667 was separated by denaturing gel electrophoresis, transferred to a nylon membrane, and probed with protein coding region of the 1,110 bp *T. brucei Nco*I-*Sal*I *AdoMetDC* fragment from the pBAce expression vector under high stringency conditions. The RNA standards are from the 1.4 - 9.5 kb RNA ladder supplied by GIBCO BRL (Gaithersburg, MD).

Northern Analysis of *T.b.brucei* Total RNA

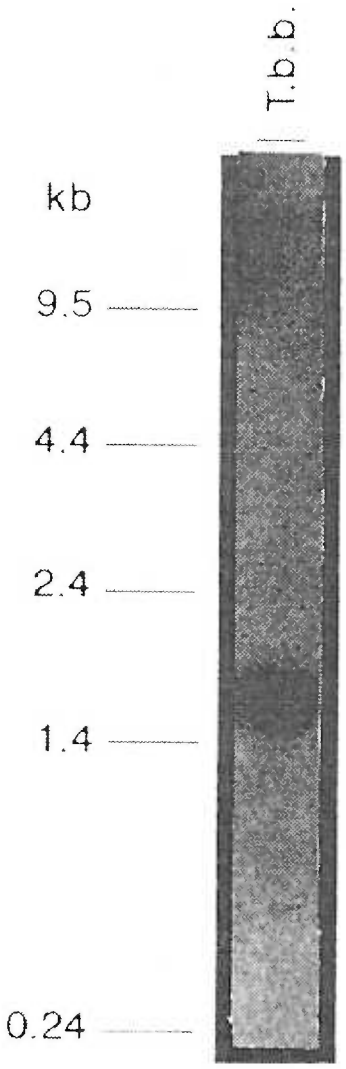


FIG. 11. ***L. donovani AdoMetDC* overexpression in *E. coli*.** The *L. donovani* *AdoMetDC*-pBAce prokaryotic expression construct was transformed into *E. coli* HT551 cells and induced by growth in the low phosphate medium described in Materials and Methods. A pBAce plasmid lacking *AdoMetDC* was transformed into HT551 cells as a negative control. After 12 hr of growth, the transformed *E. coli* were harvested, and soluble recombinant AdoMetDC enzymatic activities were measured. For reference, the activity of native *L. donovani* AdoMetDC enzyme from promastigote parasite lysates is included in the figure.

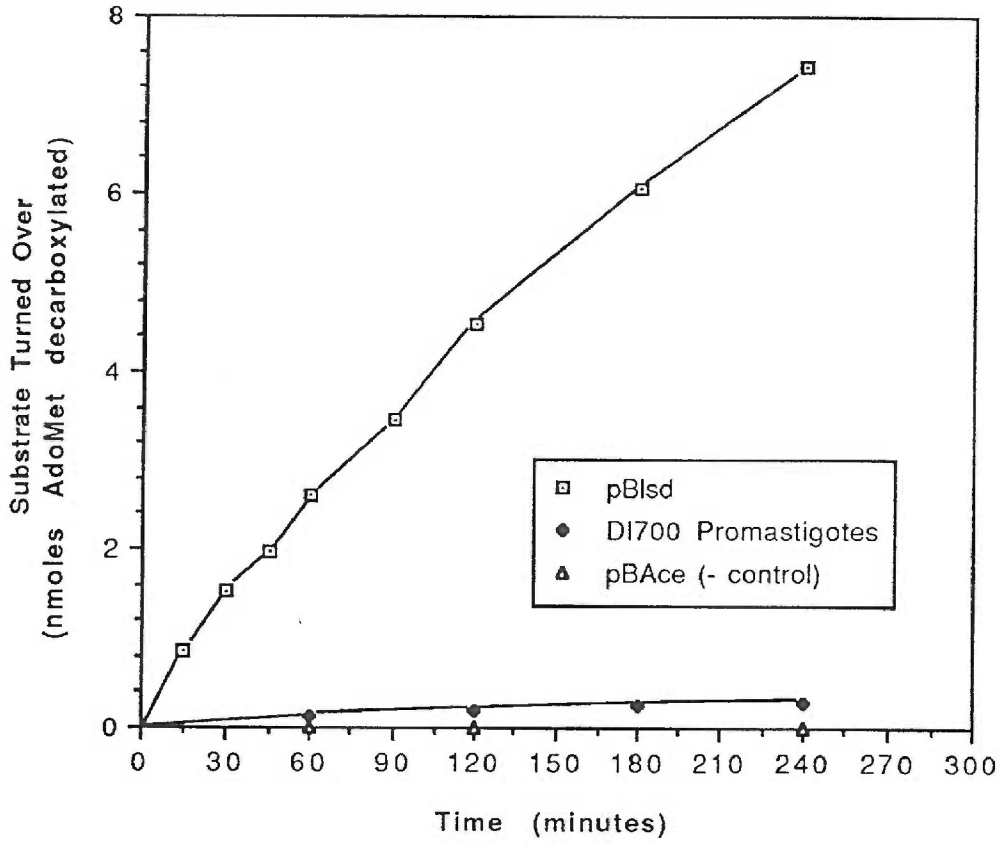

Substrate Turned Over
(nmoles AdoMet decarboxylated)
8
6
4
2
0
0
30
60
90
120
150
180
210
240
270
300
Time (minutes)
pBIsd
DI700 Promastigotes
pBAce (- control)

FIG. 12. ***T. brucei AdoMetDC* overexpression in *E. coli*.** The *T. brucei* *AdoMetDC*-pBAce prokaryotic expression construct was transformed into *E. coli* HT551 cells and induced by growth in the low phosphate medium described in Materials and Methods. A pBAce plasmid lacking *AdoMetDC* was transformed into HT551 cells as a negative control. After 12 hr of growth, the transformed *E. coli* were harvested, and soluble recombinant AdoMetDC enzymatic activities were measured. For reference, the activity of native *T. brucei* AdoMetDC enzyme from procyclic parasite lysates is included in the figure.

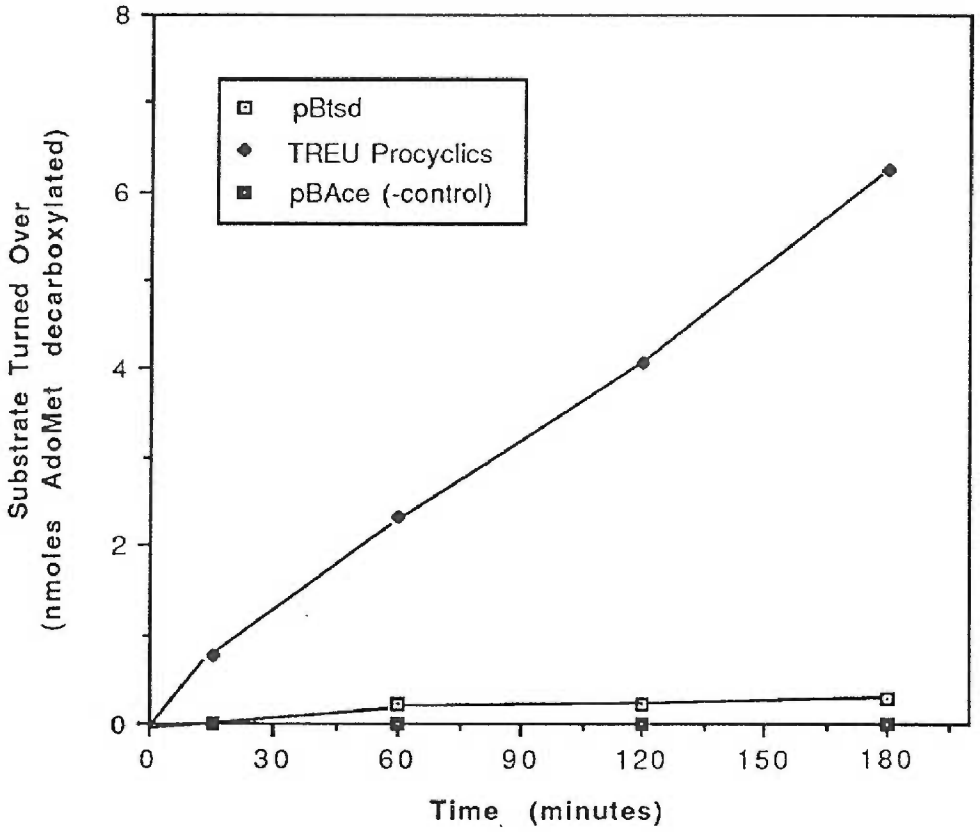

pBtsd
TREU Procyclics
pBAce (-control)
Substrate Turned Over
(nmoles AdoMet decarboxylated)
0
2
4
6
8
0
30
60
90
120
150
180
Time (minutes)

FIG. 13. **SDS polyacrylamide gel electrophoresis of *L. donovani* AdoMetDC.** As labeled in the figure, HT551 *E. coli* containing the *L. donovani AdoMetDC*-pBAce expression construct were induced as described in Materials and Methods or grown in similar medium with the addition of 1mM phosphate (uninduced) and the proteins of the crude *E. coli* extracts were subsequently fractionated by SDS polyacrylamide gel electrophoresis. ~10 μg of protein was loaded onto each lane of the SDS gel. No induced protein could be visualized by Coomassie-blue staining for the *T. brucei* construct and it was, therefore, not included in the figure.

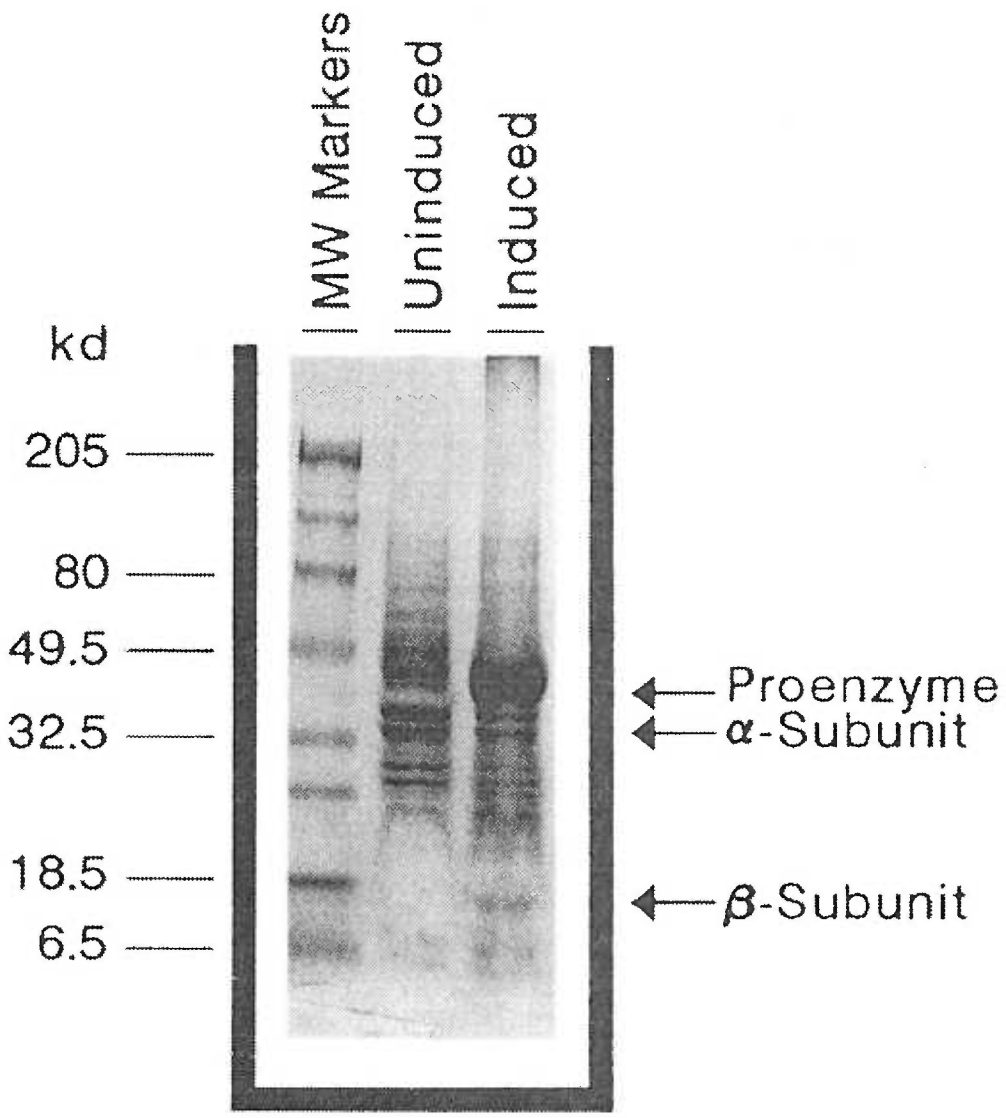

MW Markers
Uninduced
Induced
kd
205
80
49.5
32.5
18.5
6.5
Proenzyme
α-Subunit
β-Subunit

FIG. 14. **Lineweaver-Burk Analysis of Recombinant *L. donovani* AdoMetDC.** The ability of the *L. donovani* AdoMetDC enzyme to decarboxylate S-adenosylmethionine was determined at a number of substrate concentrations and the data analyzed by the method of Lineweaver-Burk. Each point represents the mean of five observations.

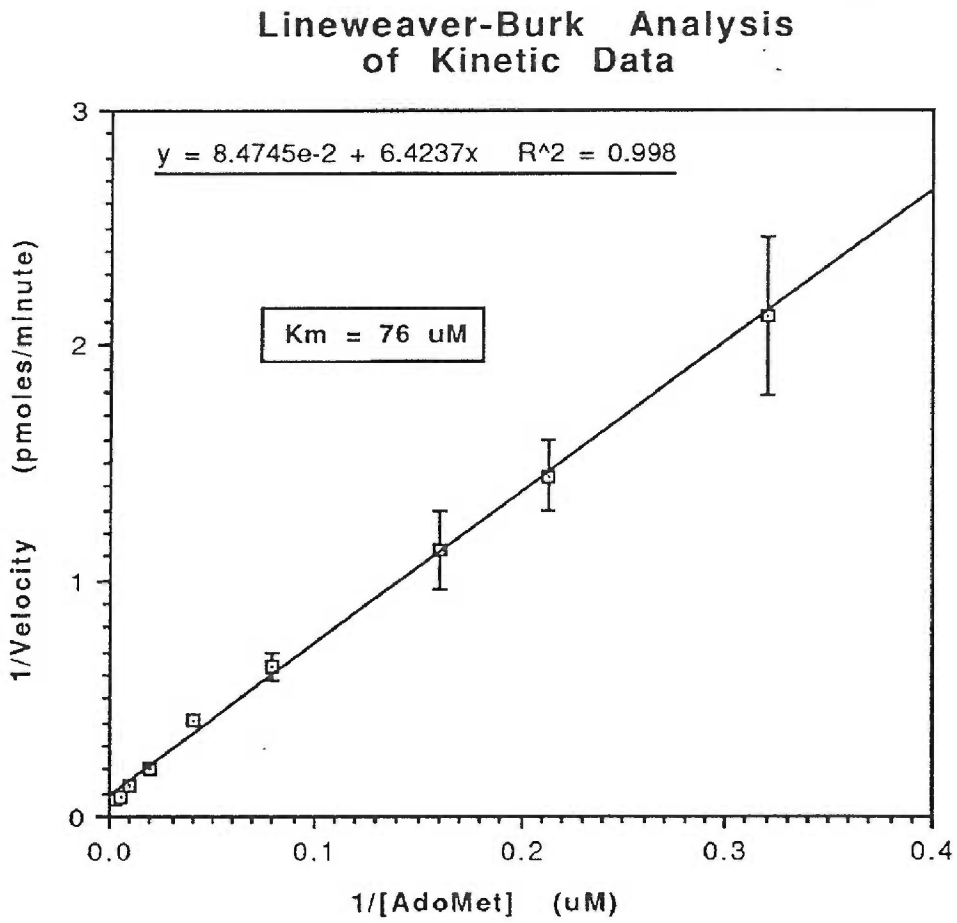

Lineweaver-Burk Analysis
of Kinetic Data
y = 8.4745e-2 + 6.4237x R^2 = 0.998
Km = 76 uM
1/Velocity (pmoles/minute)
1/[AdoMet] (uM)
0
1
2
3
0.0
0.1
0.2
0.3
0.4

FIG. 15. **Effect of Putrescine on Recombinant *L. donovani* AdoMetDC Activity.** Crude lysates of recombinant AdoMetDC were precipitated by addition of ammonium sulfate to 40% saturation. The pellet was washed three times with the ammonium sulfate solution and resuspended in putrescine-free assay buffer. Measurements were performed after two days of dialysis.

Effect of Putrescine on AdoMetDC Activity

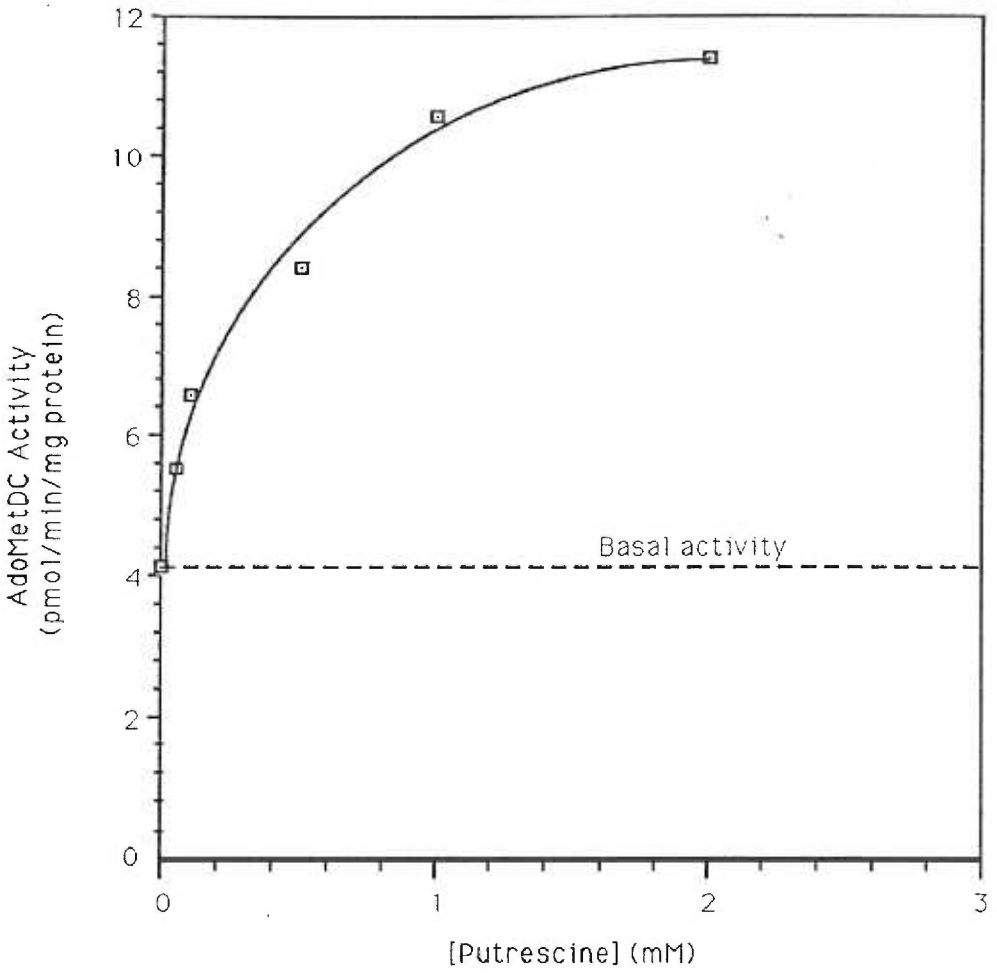


FIG. 16. **Effect of Spermidine on the Putrescine Stimulation of Recombinant *L. donovani* AdoMetDC Enzymatic Activity.** Measurements were performed on similar enzyme preparations to those in FIG. 15. Each point represents the mean of three observations.

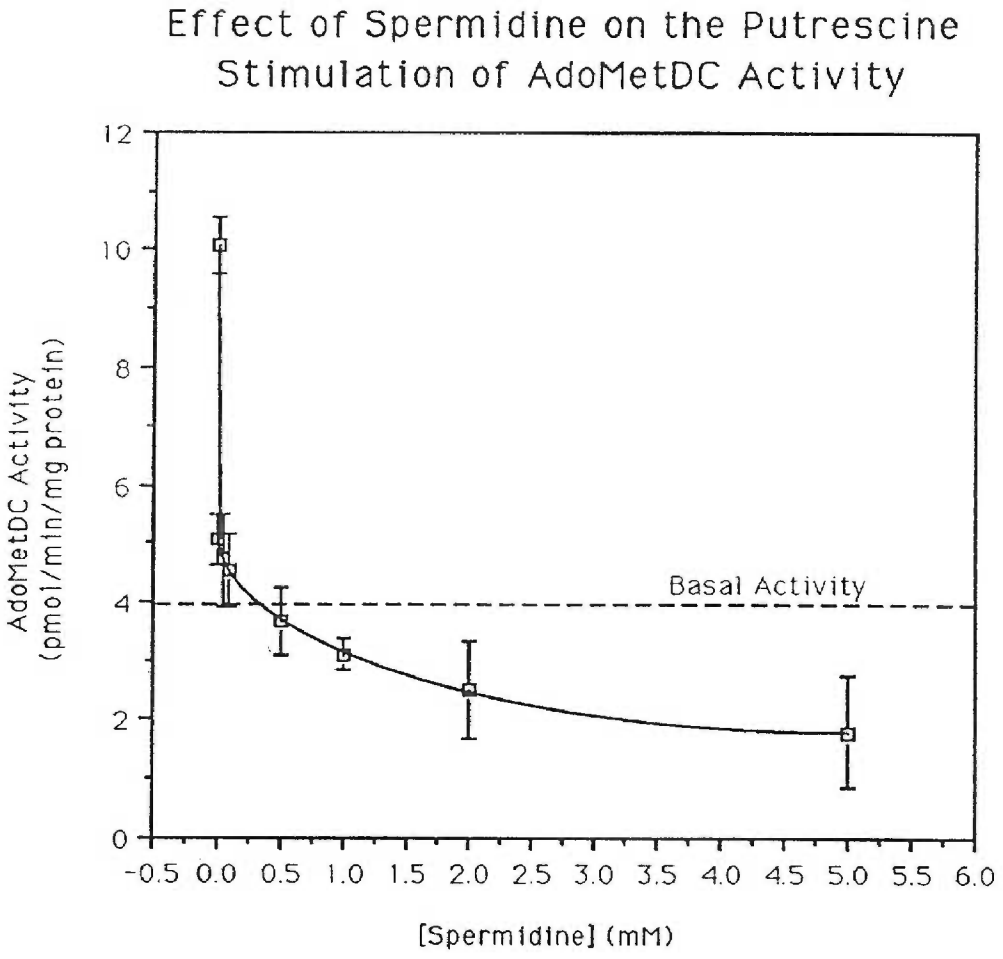

Effect of Spermidine on the Putrescine Stimulation of AdoMetDC Activity
AdoMetDC Activity (pmol/min/mg protein)
12
10
8
6
4
2
0
Basal Activity
-0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
[Spermidine] (mM)

FIG. 17. **Effect of the Polyamines, Spermidine and Spermine, on the Basal Activity and Putrescine Stimulation of Recombinant *L. donovani* AdoMetDC Activity.** Measurements were performed on similar enzyme preparations to those in FIG. 9. Each point is the mean of three observations.

Effect of Spermidine and Spermine on the Basal Activity and Putrescine Activation of AdoMetDC

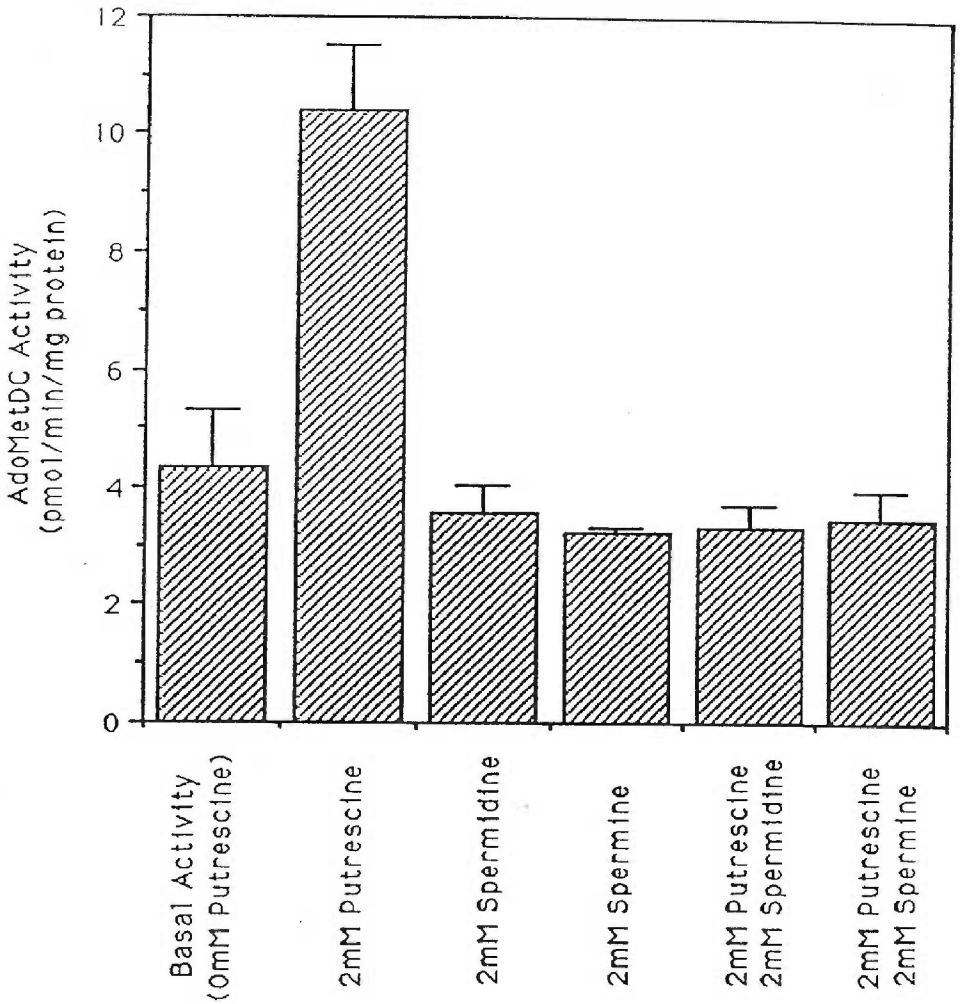


FIG. 18. **Stationary phase *L. donovani* cell lysates immunoblotted with antisera against the AdoMetDC protein.** *L. donovani* DI700 promastigotes were grown to maximum density, e.g., stationary phase, and the cell lysate (10 μg protein in each sample) was resolved by SDS-PAGE and electroblotted onto nitrocellulose. The sample was reacted with antisera against the proenzyme of the AdoMetDC enzyme.

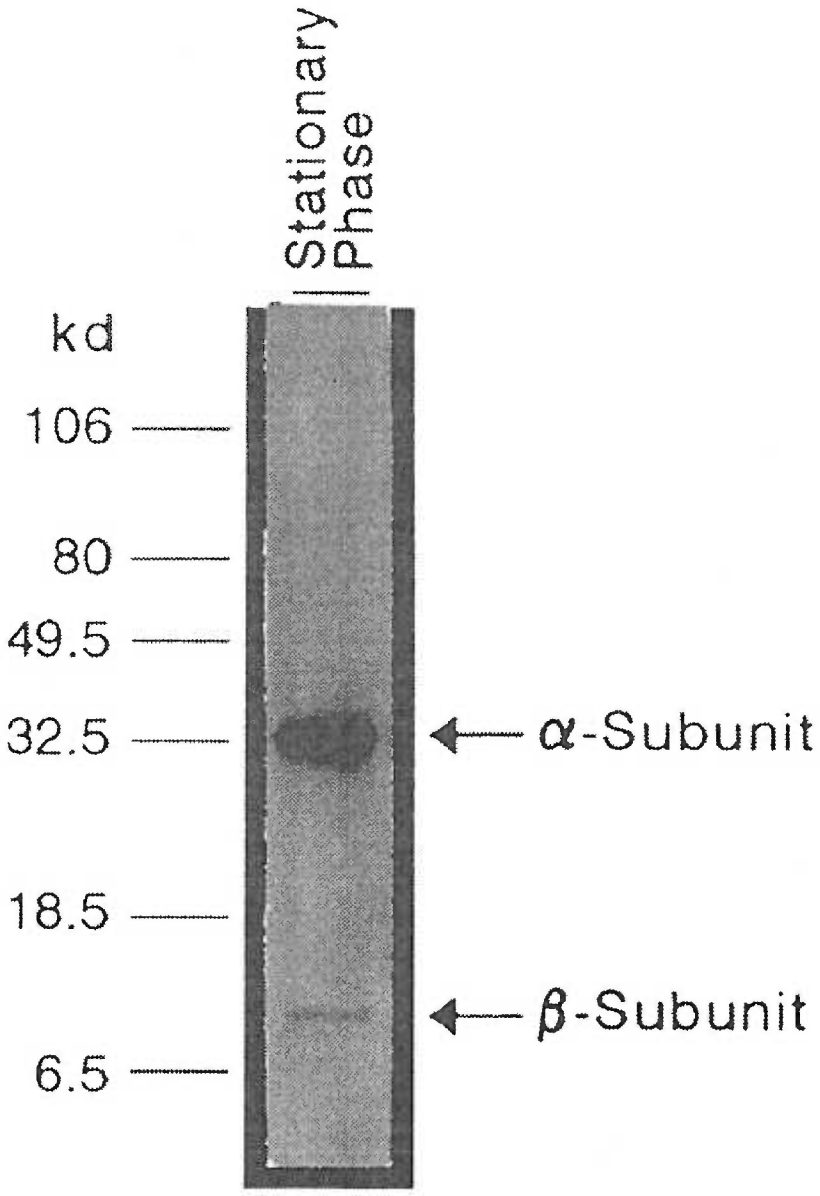

Stationary Phase
kd
106
80
49.5
32.5
18.5
6.5
α-Subunit
β-Subunit

FIG. 19. **Log phase *L. donovani* cell lysates immunoblotted with antisera against the AdoMetDC protein.** An exponentially growing culture of *L. donovani* DI700 promastigotes was grown to half-maximal density, e.g., log phase, and the cell lysate (10 μg protein in each sample) was resolved by SDS-PAGE and electroblotted onto nitrocellulose. The sample was reacted with antisera against the proenzyme of the AdoMetDC enzyme.

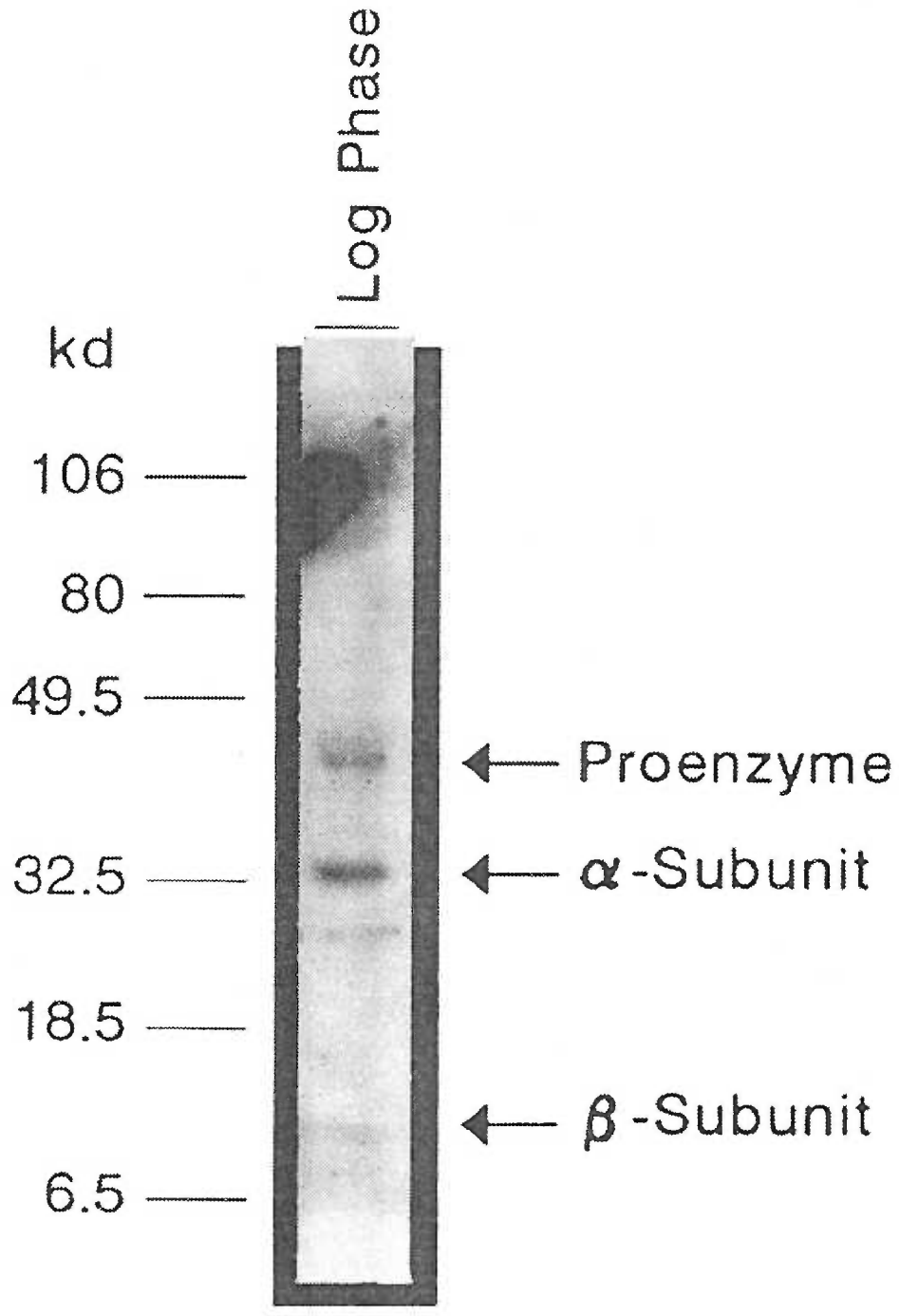
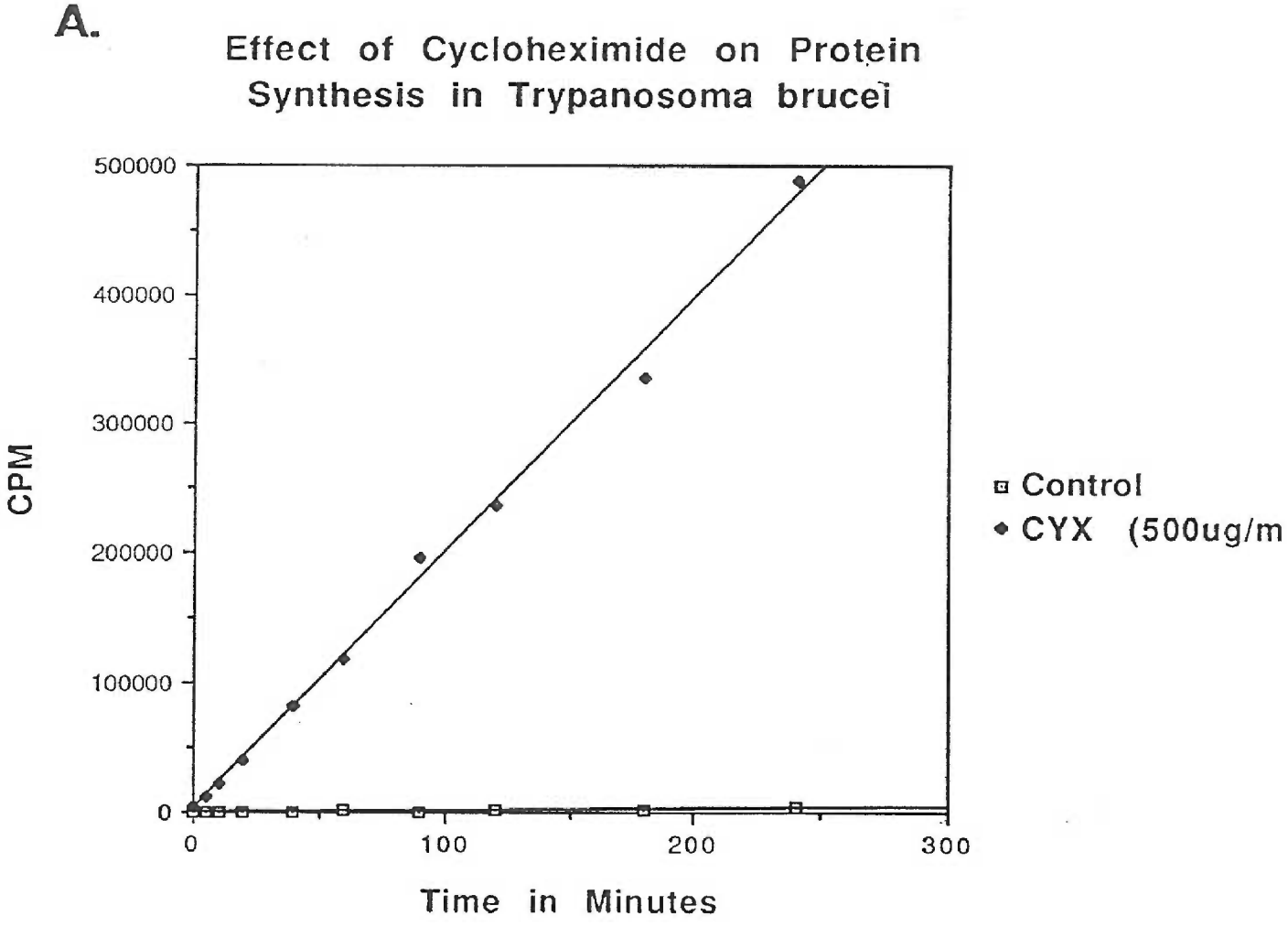

Log Phase
kd
106
80
49.5
32.5
18.5
6.5
Proenzyme
α-Subunit
β-Subunit

FIG. 20. **Measurement of the *T. brucei* AdoMetDC Enzymatic Half-life.** A, [^{35}S]methionine incorporation of *T. brucei* procyclic cells. □, no drug, control. ♦, incubated in the presence of 500 μg/ml cycloheximide. B, AdoMetDC enzymatic activity (nmol CO_2/h/mg protein) following incubation with 500 μg/ml cycloheximide. For the determination of enzymatic activity each point represents the mean of two observations.

A.


Effect of Cycloheximide on Protein Synthesis in Trypanosoma brucei
500000
400000
300000
200000
100000
0
CPM
0
100
200
300
Time in Minutes
Control
CYX (500ug/m

B.

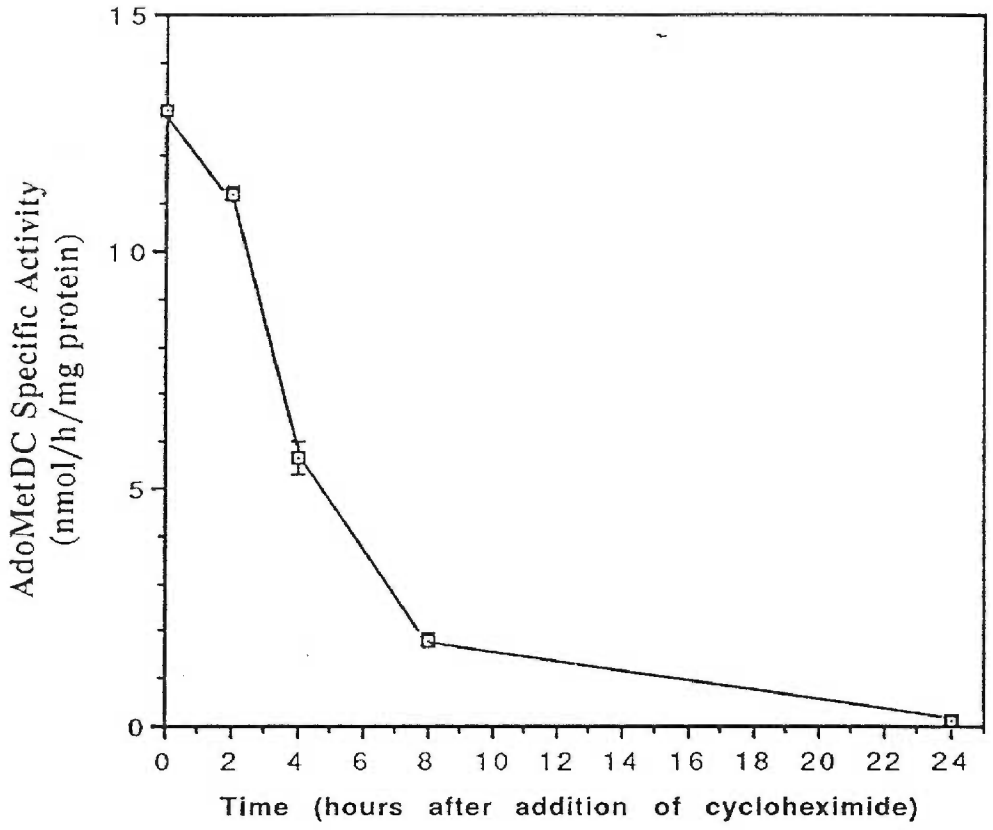

AdoMetDC Specific Activity
(nmol/h/mg protein)
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours after addition of cycloheximide)

FIG. 21. **Semi-Logarithmic Plot of the *T. brucei* AdoMetDC Enzymatic Half-life.** The data from FIG. 20. were plotted semi-logarithmically to determine the AdoMetDC enzymatic half-life. The slope of the line multiplied by the natural logarithm of 10 was found to be 3.7 hr.

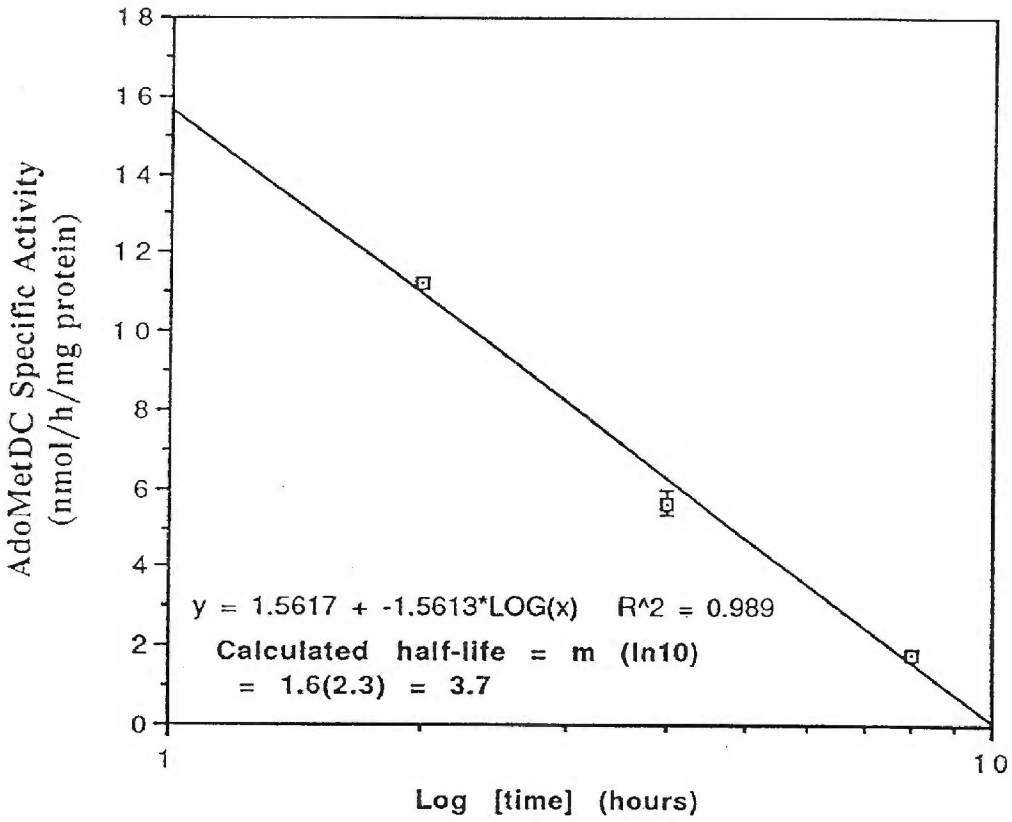
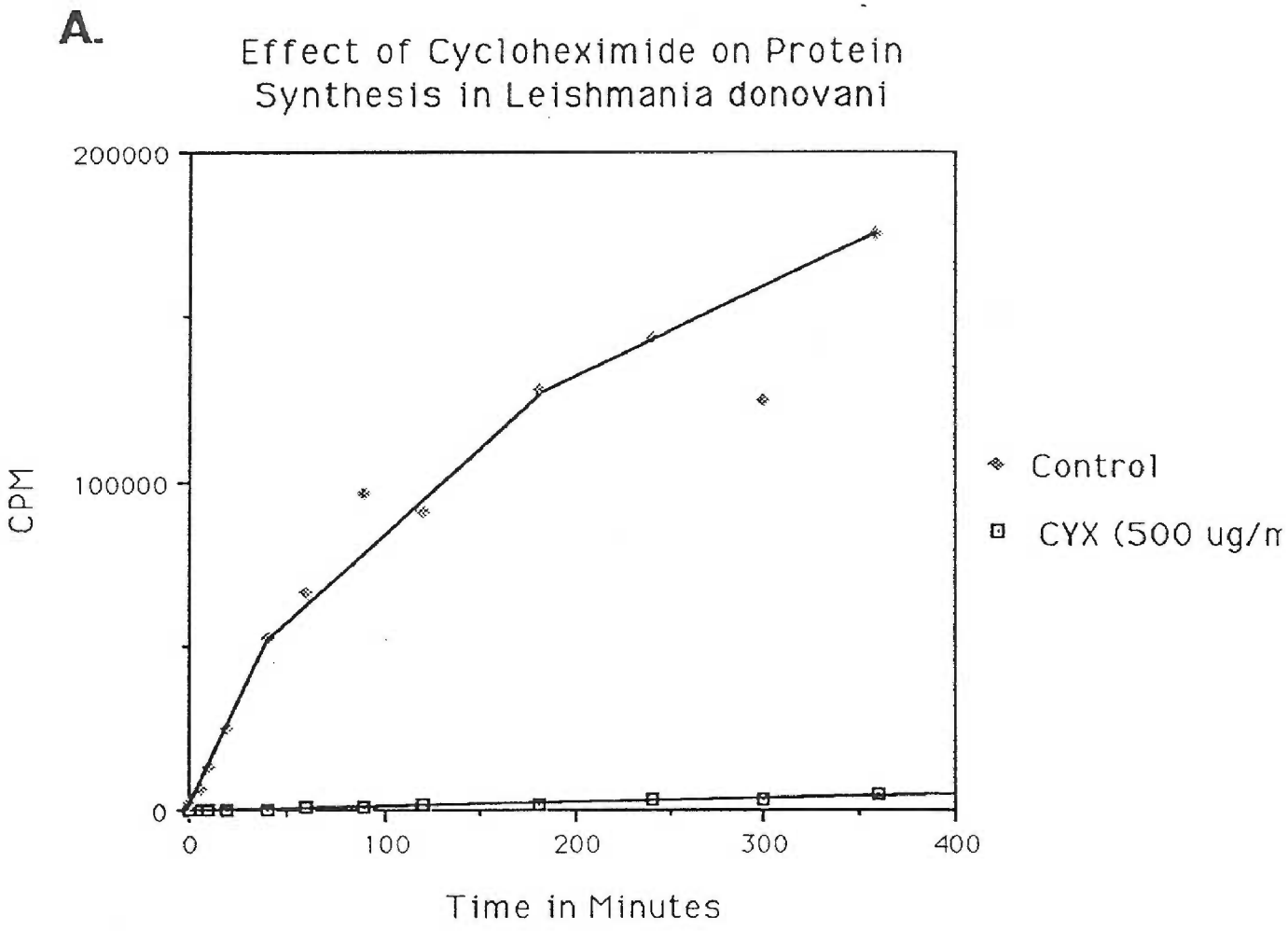

AdoMetDC Specific Activity
(nmol/h/mg protein)
18
16
14
12
10
8
6
4
2
0
1
10
y = 1.5617 + -1.5613*LOG(x) R^2 = 0.989
Calculated half-life = m (ln10)
= 1.6(2.3) = 3.7
Log [time] (hours)

FIG. 22. **Measurement of the *L. donovani* AdoMetDC Enzymatic Half-life.** A, [^{35}S]methionine incorporation of *L. donovani* procyclic cells. □, no drug, control. ♦, incubated in the presence of 500 μg/ml cycloheximide. B, AdoMetDC enzymatic activity (pmol CO_2/min/mg protein) following incubation with 500 μg/ml cycloheximide. For the determination of enzymatic activity each point represents the mean of two observations.

A.


Effect of Cycloheximide on Protein Synthesis in Leishmania donovani
200000
100000
0
CPM
0
100
200
300
400
Time in Minutes
Control
CYX (500 ug/m

B.

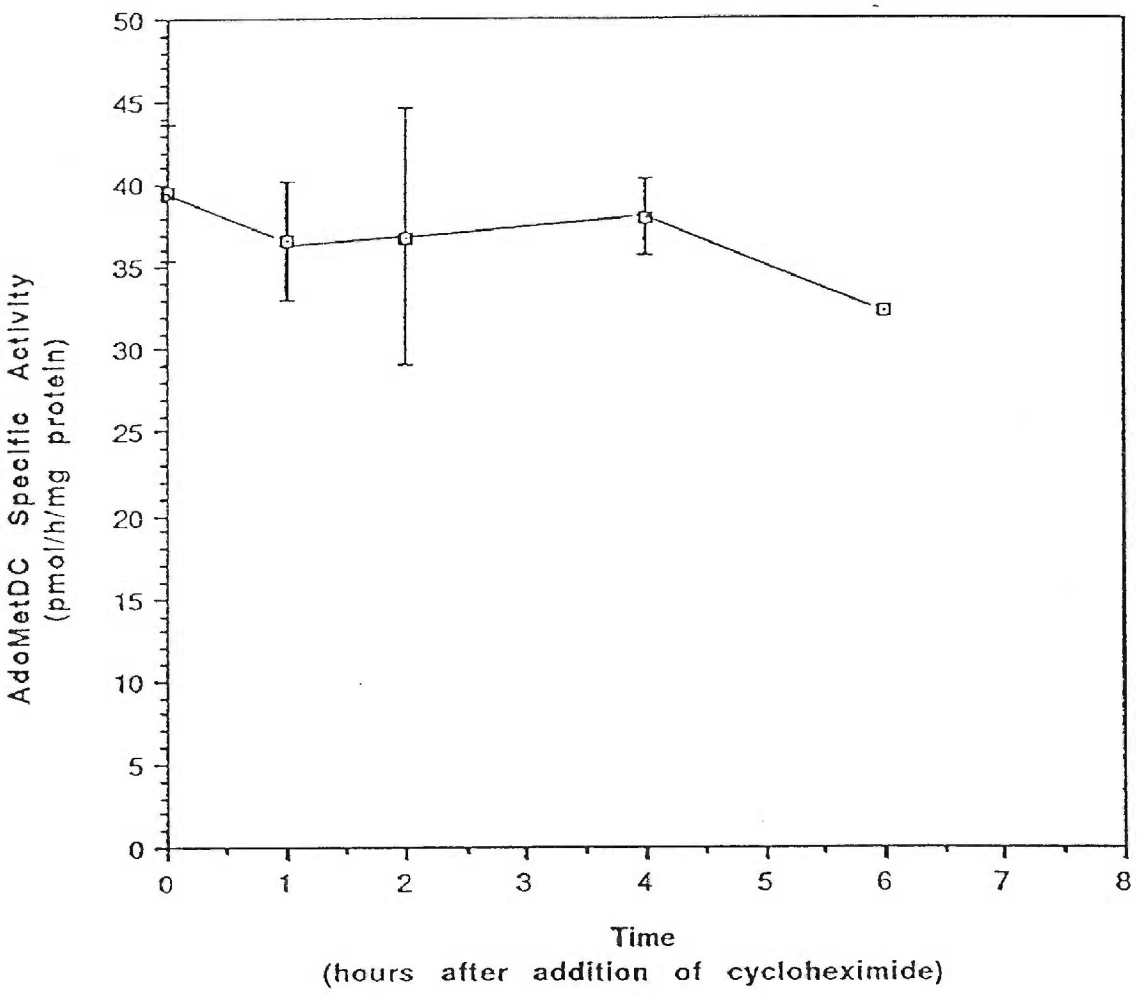

AdoMetDC Specific Activity
(pmol/h/mg protein)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Time
(hours after addition of cycloheximide)

FIG. 23. **MDL 73811 Toxicity Toward *L. donovani* DI700 (wild-type) and MDL 1000 procyclic cells.** The observations are plotted as a percentage of the growth in control wells lacking MDL 73811. 5 similar experiments were performed with identical results.

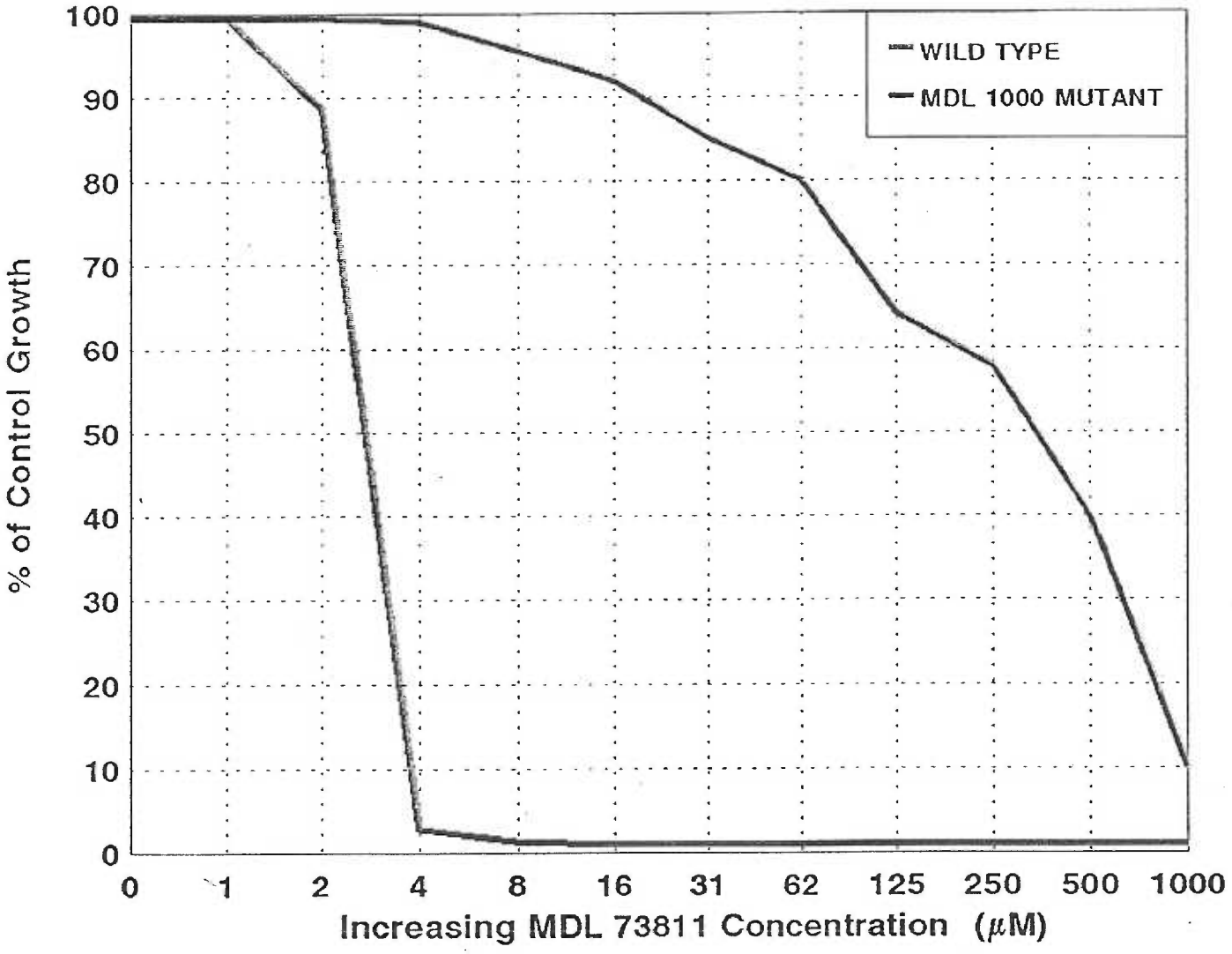

WILD TYPE
MDL 1000 MUTANT
% of Control Growth
100
90
80
70
60
50
40
30
20
10
0
0
1
2
4
8
16
31
62
125
250
500
1000
Increasing MDL 73811 Concentration (μM)

FIG. 24. **Southern Blot Analysis of *L. donovani* DI700 and MDL 1000 Genomic DNA.** Genomic DNA was isolated from DI700 and MDL 1000 cells that had been removed from MDL 73811 for 5 days. The gDNA was digested with *Xba*I, separated by 0.8% agarose gel electrophoresis, and probed with the protein coding region of the *L. donovani AdoMetDC* gene. Similar digests and analyses with *Sac*I, which cuts once within the coding sequence of the gene, and *Sal*I, which does not cut, yielded similar results.

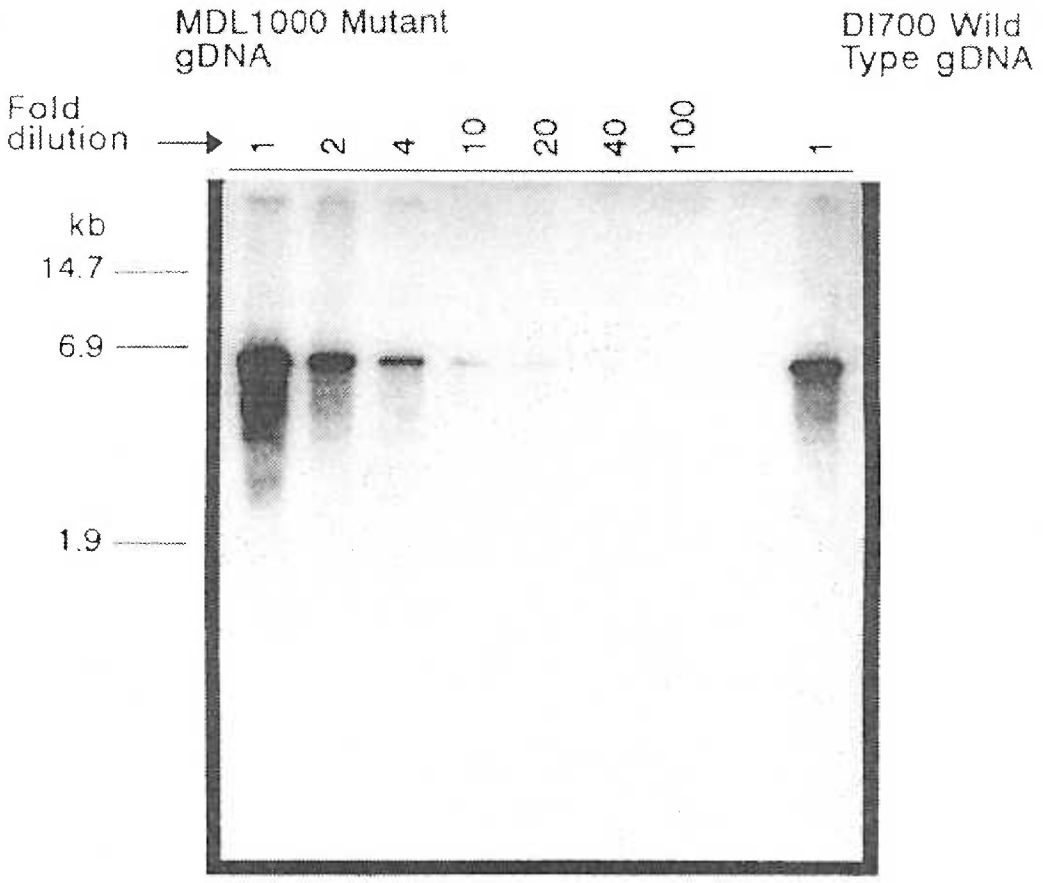
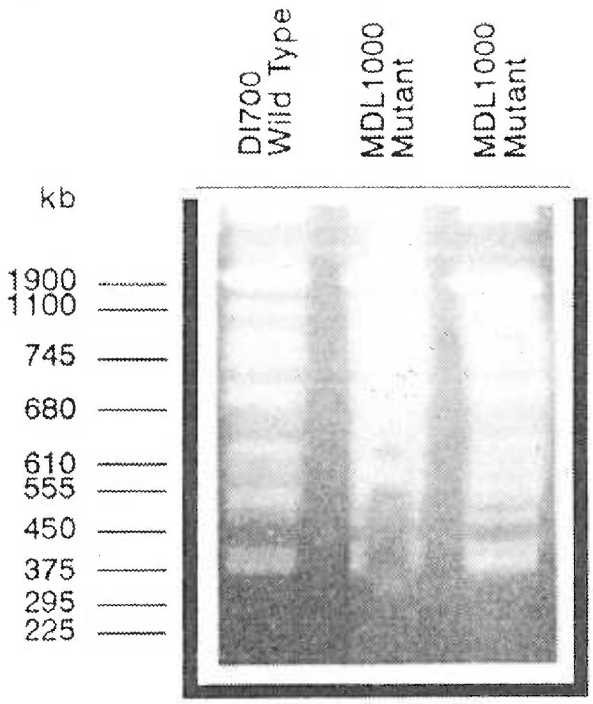

MDL1000 Mutant
gDNA
DI700 Wild
Type gDNA
Fold
dilution
1
2
4
10
20
40
100
1
kb
14.7
6.9
1.9

FIG 25. **Pulsed Field Gel Electrophoresis and Southern Blot Analysis of *L. donovani* DI700 and MDL 1000 Chromosomes.** Chromosomal location of the *L. donovani AdoMetDC* gene in DI700 and MDL 1000 cells. A pulsed field gel of DI700 and MDL 1000 chromosomes was obtained with a pulse time of 60 sec, transferred to a nylon membrane, and probed with the protein coding region of the AdoMetDC gene. The M_r's of leishmanial DNAs were estimated by comparing their mobilities to those of yeast chromosomal markers from *S. cerevisiae* strain YNN295.


DI700
Wild Type
MDL1000
Mutant
MDL1000
Mutant
kb
1900
1100
745
680
610
555
450
375
295
225

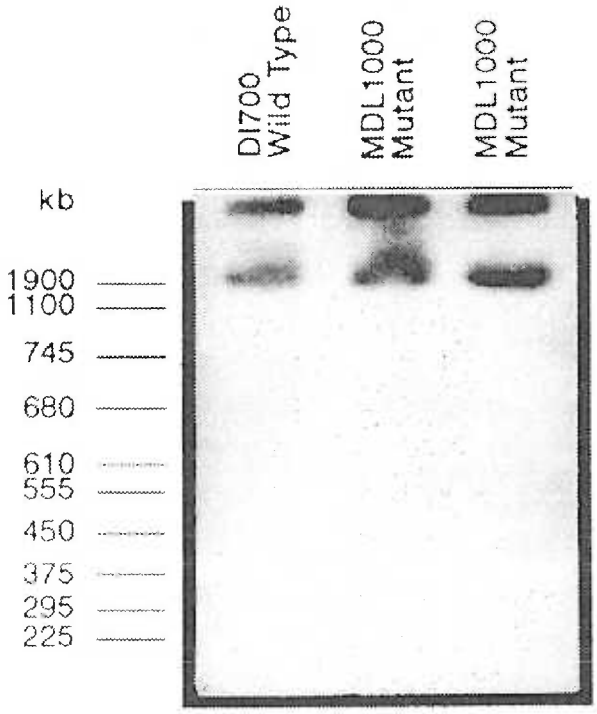

DI700
Wild Type
MDL1000
Mutant
MDL1000
Mutant
kb
1900
1100
745
680
610
555
450
375
295
225

FIG 26. **Northern Blot Analysis of *L. donovani* DI700 and MDL 1000 Total RNA.** The levels of AdoMetDC transcript expressed in DI700 and MDL 1000 cells were assessed by Northern blotting. Total RNA was fractionated on formaldehyde/denaturing gels, transferred to nylon membranes, and hybridized to the radio-labeled coding sequence of the *L. donovani AdoMetDC* gene.

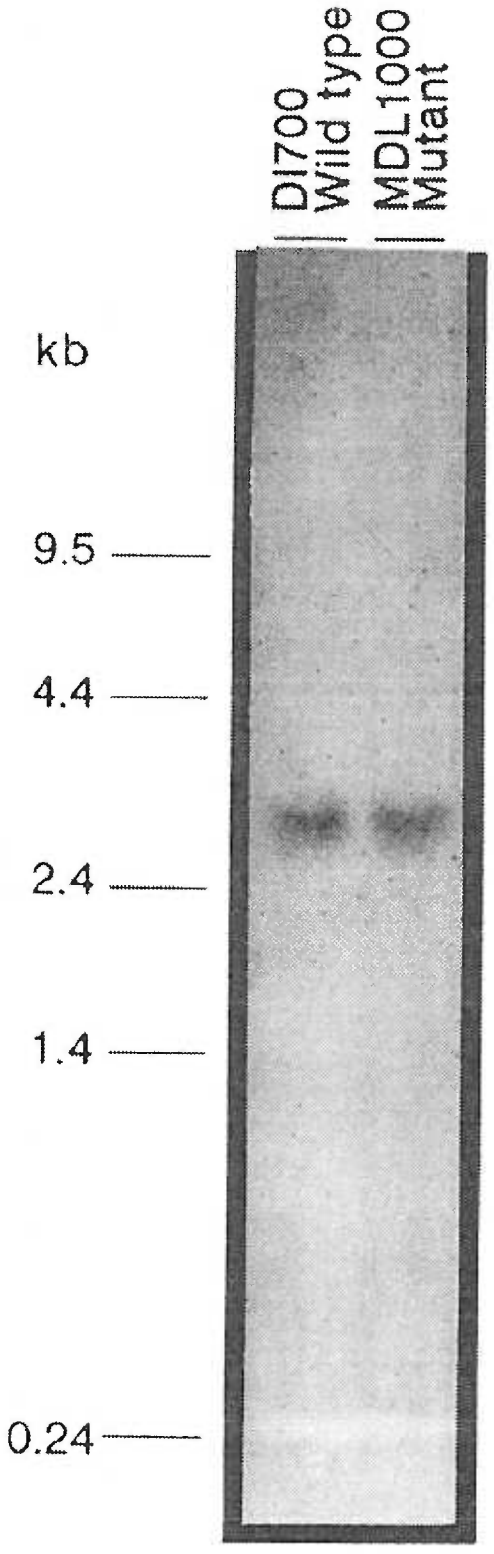

DI700
Wild type
MDL1000
Mutant
kb
9.5
4.4
2.4
1.4
0.24

FIG. 27. **AdoMetDC Enzymatic Activity in *L. donovani* DI700 and MDL 1000 cells.** The specific activity of AdoMetDC in lysates of DI700 and MDL 1000 cells were measured as described in Materials and Methods. Extracts of the MDL 1000 cells were prepared 5 days after resuspension in nonselective medium in order to allow for the synthesis of new AdoMetDC protein in the absence of MDL 73811.

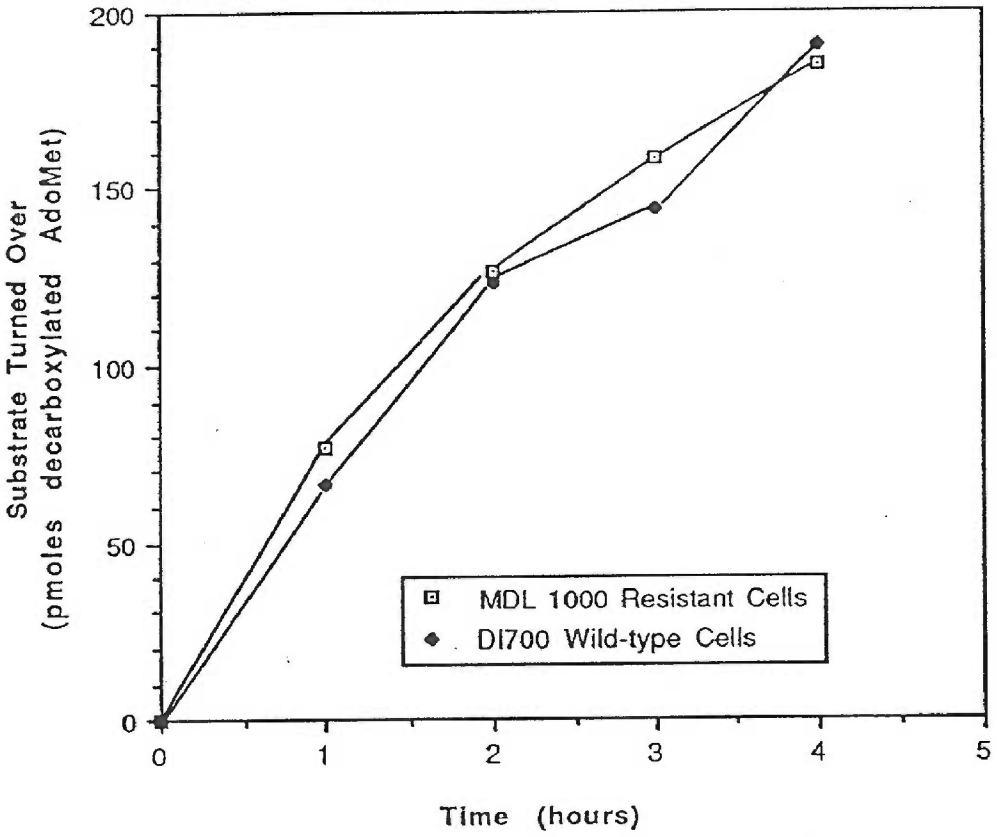

Substrate Turned Over
(pmoles decarboxylated AdoMet)
200
150
100
50
0
0
1
2
3
4
5
Time (hours)
MDL 1000 Resistant Cells
DI700 Wild-type Cells

FIG 28. **Sensitivity of AdoMetDC activity to inhibition by MDL 73811 in *L. donovani* DI700 and MDL 1000 cells.** The specific activity of AdoMetDC in lysates of DI700 and MDL 1000 cells were measured in the presence of increasing MDL 73811 drug concentrations. The specific activity of AdoMetDC in lysates of DI700 and MDL 1000 cells were measured as described in Materials and Methods except that the concentration of S-adenosylmethionine was limited to 50 μM in each of the experiments. Extracts of the MDL 1000 cells were prepared 5 days after resuspension in nonselective medium in order to allow for the synthesis of new AdoMetDC protein in the absence of MDL 73811.

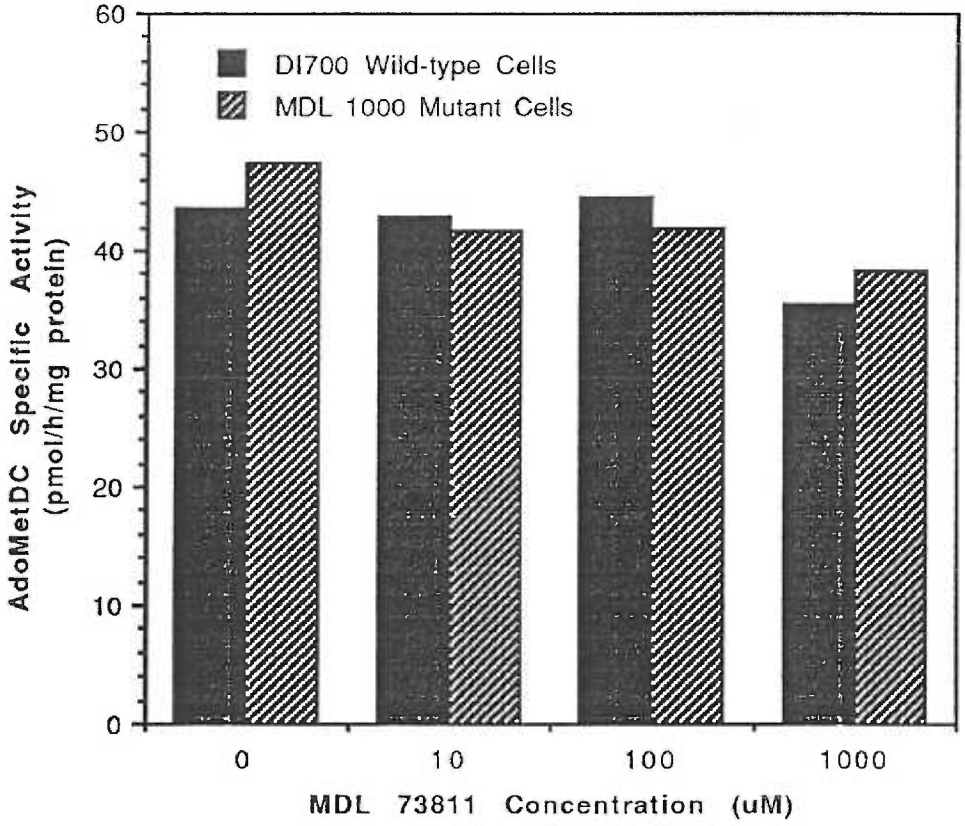

DI700 Wild-type Cells
MDL 1000 Mutant Cells
AdoMetDC Specific Activity
(pmol/h/mg protein)
60
50
40
30
20
10
0
0
10
100
1000
MDL 73811 Concentration (uM)

FIG. 29. **Log phase *L. donovani* DI700 and MDL 1000 cell lysates immunoblotted with antisera against the AdoMetDC protein.** An exponentially growing culture of *L. donovani* DI700 and MDL 1000 promastigotes were grown to half-maximal density, e.g., log phase, and the cell lysates (10 μg protein in each sample) was resolved by SDS-PAGE and electroblotted onto nitrocellulose. The samples were reacted with antisera against the proenzyme of the AdoMetDC enzyme.

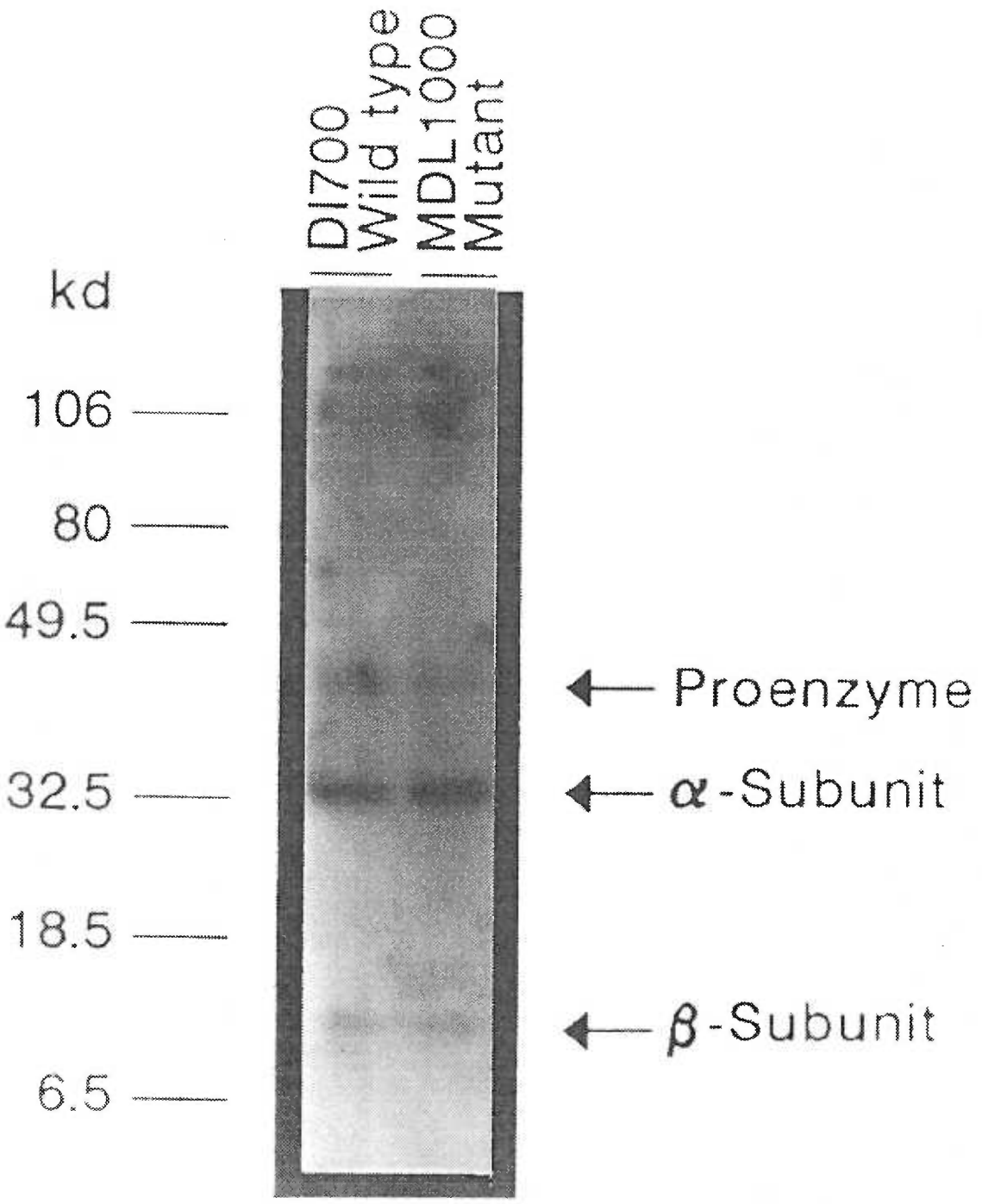
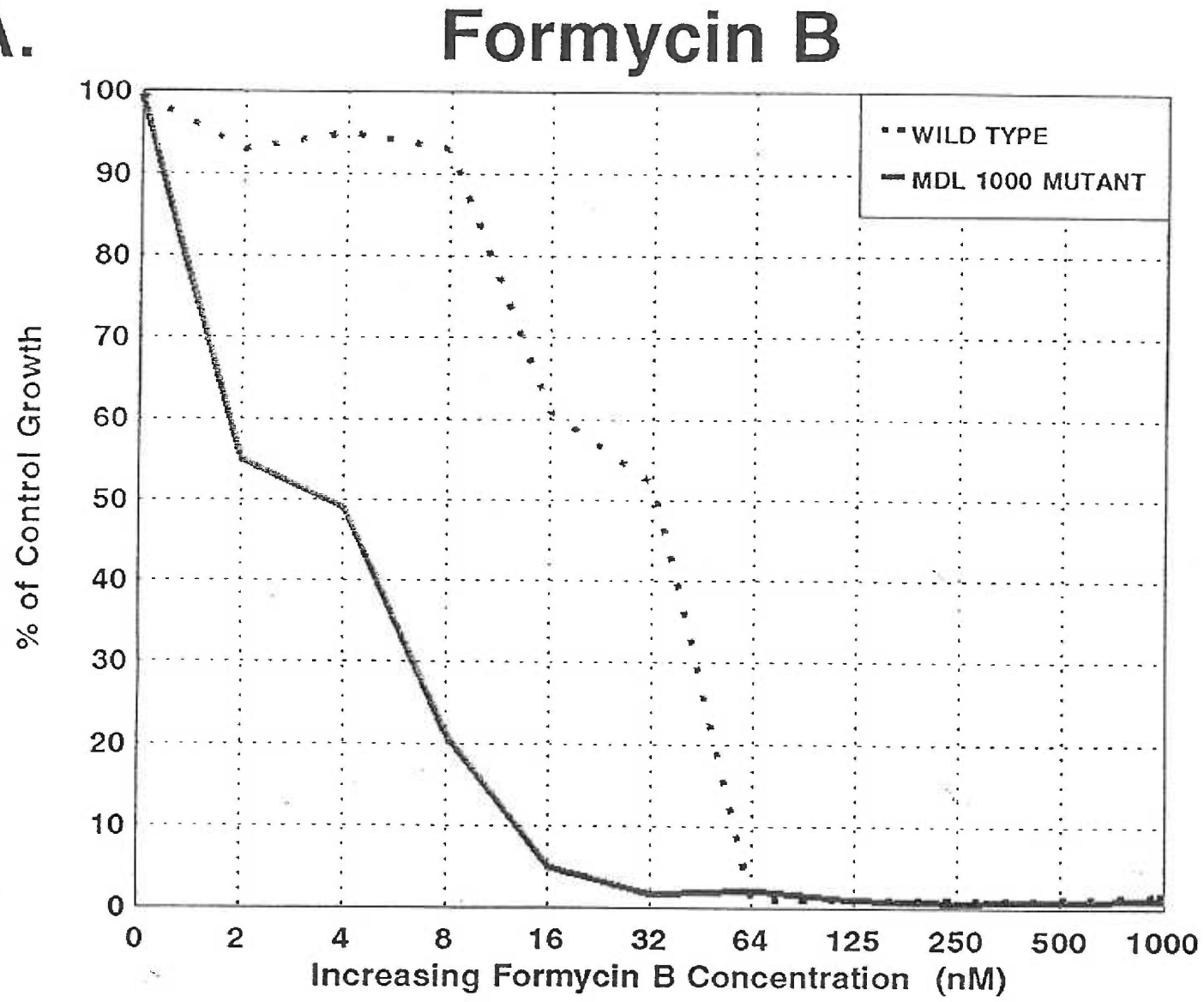
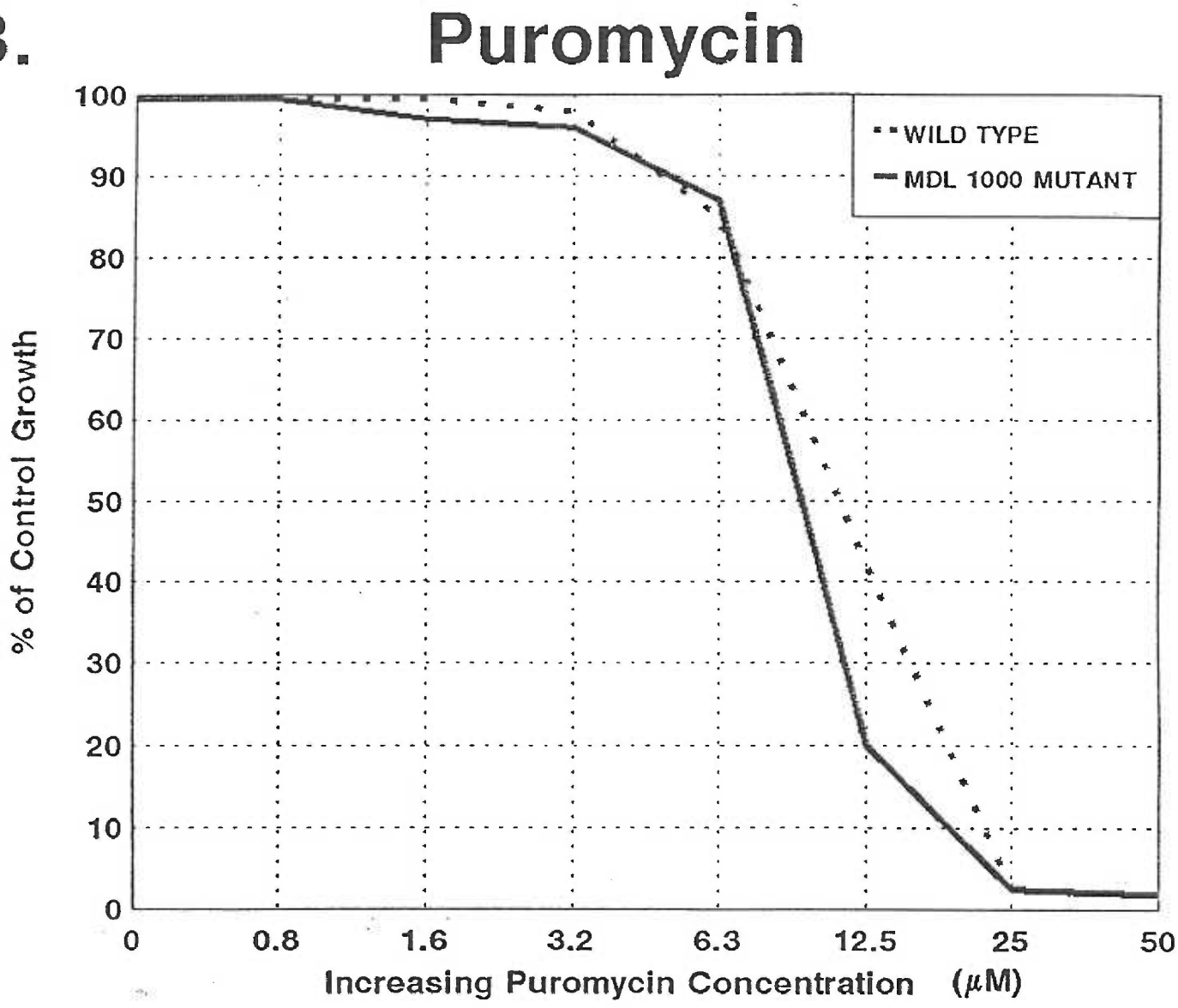

DI700
Wild type
MDL1000
Mutant
kd
106
80
49.5
32.5
18.5
6.5
Proenzyme
α-Subunit
β-Subunit

FIG. 30. **Effect of Toxic Purine Analogs on MDL 1000 Mutant Cells and DI700 Wild-Type Cells.** MDL 73811 is a purine analog and since it is theoretically possible that resistance to MDL 73811 results from an alteration in transport activity, the sensitivity of the mutant cell line to toxic purine analogs was assayed as an indicator of the patency of the purine transporter: A, Formycin A; B, Puromycin; C, Tubercidin.

A.


Formycin B
WILD TYPE
MDL 1000 MUTANT
% of Control Growth
100
90
80
70
60
50
40
30
20
10
0
0
2
4
8
16
32
64
125
250
500
1000
Increasing Formycin B Concentration (nM)

B.


Puromycin
WILD TYPE
MDL 1000 MUTANT
% of Control Growth
100
90
80
70
60
50
40
30
20
10
0
0
0.8
1.6
3.2
6.3
12.5
25
50
Increasing Puromycin Concentration (μM)

C.

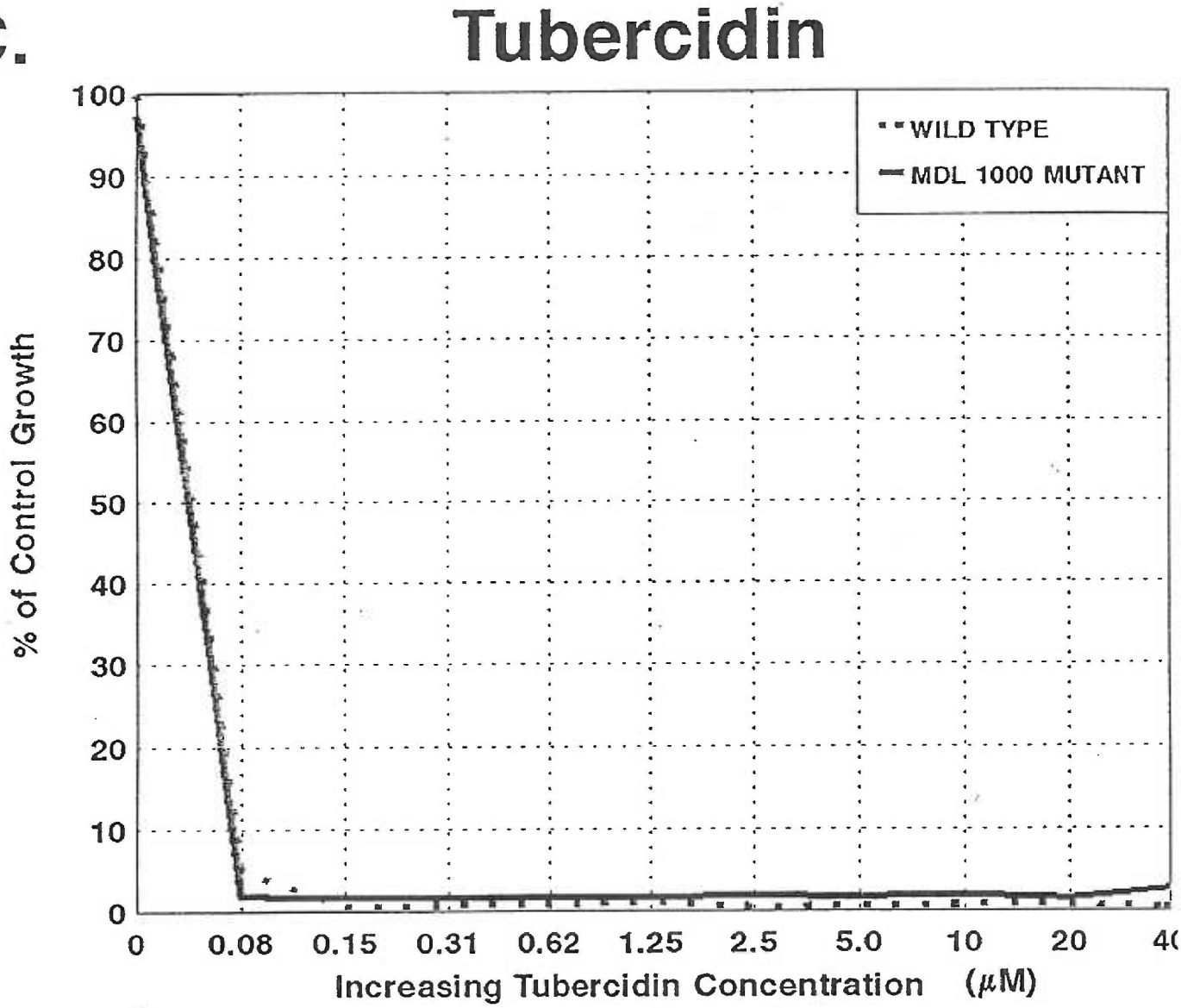

Tubercidin
WILD TYPE
MDL 1000 MUTANT
% of Control Growth
100
90
80
70
60
50
40
30
20
10
0
0
0.08
0.15
0.31
0.62
1.25
2.5
5.0
10
20
Increasing Tubercidin Concentration (μM)

Table I. **Comparison of the Predicted AdoMetDC Amino Acid Sequences from *L. donovani, T. brucei, S. cerevisiae* (Yeast), Human, Potato, and *E. coli*.** The percent identity between the various reported AdoMetDC protein sequences were compared in a pairwise manner after alignment of their sequences as described in Materials and Methods.

TABLE I

Comparison of AdoMetDC amino acid sequences

	L.donovani	Yeast	Human	Potato	E.coli
T.brucei	63%	22%	26%	27%	11%
L.donovani	-	22%	27%	28%	10%
Yeast	-	-	34%	27%	11%
Human	-	-	-	33%	10%
Potato	-	-	-	-	8%

CONCLUSIONS AND FUTURE DIRECTIONS

Because the AdoMetDC enzymes of *T. brucei* and *L. donovani* are likely chemotherapeutic targets, the overall goal of this thesis was to identify their respective genes and to characterize the regulation of these enzymes and their role in the control of the polyamine biosynthetic pathway.

The isolation and molecular characterization of the *L. donovani* and *T. brucei AdoMetDC* genes and the high level expression of recombinant *L. donovani AdoMetDC* have allowed us to make significant progress towards this goal. Biochemical characterization of the recombinant *L. donovani* AdoMetDC has shown that its K_m for adenosylmethionine of 76 μM is comparable to the reported K_ms of other eukaryotic AdoMetDC enzymes. Kinetic studies have also shown that the enzyme is controlled in a classic biochemical fashion in that its enzymatic activity is stimulated by putrescine, a polyamine intermediate, and that this stimulation, as well as the basal activity of AdoMetDC, is inhibited by spermidine, the end product of the polyamine pathway in kinetoplastid parasites. Studies on the native trypanosomal enzyme have also shown this to be the case and in this respect, the parasite enzymes are remarkably similar to their human counterpart. Furthermore, from the alignment of the predicted amino acid sequences of the human and parasite proteins and reported mutational studies on the human enzyme, we can infer that the processing of the proenzyme to the α- and β-subunits of the active enzyme is probably stimulated by putrescine. Despite these kinetic and regulatory similarities we now know that the human and parasite enzymes are highly divergent at the amino acid sequence level and this will

perhaps, in the future, lead to the discovery of structural differences which can be exploited for drug design. Along these same lines, the fact that the leishmanial AdoMetDC is strongly inhibited by spermidine, coupled with the ability of *Leishmania* to transport polyamines, may ultimately lead to the development of rational chemotherapeutic strategies that exploit these attributes. Further, the characterization of the trypanosomal AdoMetDC turnover rate as substantially greater than that of the mammalian enzyme could partially explain why trypanocidal inhibitors of AdoMetDC, such as MDL 73811, preferentially harm the parasite, thereby effecting cures of murine models of African trypanosomiasis.

One of the specific aims of this thesis was the high level expression of recombinant *T. brucei AdoMetDC* and its purification. The disappointingly low levels of recombinant expression have made this impractical at this time. However, it is important to note that the enzyme is processed correctly in bacteria; thus, if the expression could be increased by either using a more powerful promoter or by changing the *Nco*I site to a *Nde*I site which is purported to be translated more efficiently by bacterial systems, then one could easily purify large amounts of recombinant protein over nickel agarose columns using a hexahistidine tag. The PET vector system, which uses the T7 bacteriophage promoter and also encodes a hexahistidine tag incorporates all of these positive attributes; however, the construct used for expression in my experiments contained a 5' leader sequence, e.g., the PelB leader, that encodes for secretion of the recombinant protein into the bacterial peri-plasmic space. This construct was obviously expressed, but was extremely toxic and subject to recombination. If the PelB leader sequence were removed or a different plasmid with a similar T7 promoter were utilized, I am certain that one could obtain high level expression of the *T. brucei*

AdoMetDC. Beyond these technical difficulties, the isolation and characterization of the *T. brucei AdoMetDC* gene and the knowledge of the enzyme's predicted primary structure have provided useful information regarding the regulation of the AdoMetDC enzyme and its role in the control of the polyamine biosynthetic pathway. In addition, should the crystallographic structures of the *L. donovani* or human AdoMetDC enzymes be solved, the knowledge of the amino acid sequence of the *T. brucei* enzyme will enable its tertiary structure to be modeled and thereby aid in the rational design of therapeutic inhibitors. Thus, the inability to obtain high levels of expression of recombinant trypanosomal *AdoMetDC* is not an insurmountable hurdle towards the rational design of specific inhibitors that target this enzyme.

Finally, in order to examine the effects of the experimental AdoMetDC inhibitor, MDL 73811, on *Leishmania*, a drug resistant strain, MDL 1000, was generated that was at least 1000-fold less sensitive to the toxic effects of MDL 73811 than wild-type *L. donovani* parasites. Since the molecular characterization of the resistant strain showed no alteration of the AdoMetDC gene locus, no transcriptional or translational activation, and no alteration in the kinetic characteristics of AdoMetDC enzymatic activity despite a 250-fold decrease in sensitivity to the MDL 73811 drug, our data lead us to conclude that the AdoMetDC enzyme is not the primary target of the MDL 73811 compound in *L. donovani*. With this in mind, it is significant that the initial report of the successful treatment of murine trypanosomiasis with MDL 73811 noted that the curative effects of the drug could not be blocked by the infusion of putrescine, spermidine, or spermine, which should theoretically circumvent the blockade and cause further parasitemia, as occurs when this

protocol is employed during the administration of curative levels of DFMO. To determine if the trypanosomal AdoMetDC enzyme is actually a primary target of MDL 73811, a drug resistant *T. brucei* cell line could be developed and examined both biochemically and at the molecular level using the *T. brucei AdoMetDC* gene as a molecular probe.

In summary, we have answered a number of relevant molecular and biochemical questions regarding the regulatory role of the AdoMetDC enzyme in the polyamine biosynthetic pathway of *Leishmania* and *T. brucei*. As a byproduct of this work we have also provided a number of tools, e.g., the cloned parasite *AdoMetDC* genes, the recombinant *L. donovani AdoMetDC* expression system, and the AdoMetDC-specific antibodies, which should be useful to others in the continued study of these fascinating and medically relevant organisms.

APPENDIX A

Molecular phylogenic analysis of *Leishmania donovani*, *Trypanosoma brucei*, and *Trypanosoma cruzi* utilizing the AdoMetDC enzyme as a model protein.

Taxonomy, or the science of classification, is one of the oldest of the sciences, having its origins in the Greek word "taxis" or arrangement. For several millenia the art of taxonomy went relatively unchanged; however, with the advent of Darwin's evolutionary theories, an almost violent revolution embroiled the faculty and it has since come to be almost synonymous in the biological sciences with phylogeny, or the study of the evolutionary development of groups of organisms. For the better part of a century phylogenic classification has involved the classification and grouping of organisms according to phenotypic and morphological similarities, stimulating hours and volumes of entertaining and sometimes acrimonious debate as to which characteristics were the most significant. Now, almost 100 years after Darwin's death, the science of phylogeny has itself been revolutionized by our ability to obtain the nucleotide sequences of genes and thereby define evolutionary distances, not by differences in appearances, but by mathematical terms which can be analyzed and in turn yield valuable information (1). Thus, it is now known that humans and chimpanzees are probably more closely related than either is to the gorilla (2) and that contemporary humanity likely descended from an unusually successful common ancestor on the African continent between 150,000 and 200,000 years ago (3).

The current model of molecular evolution is that mutations, at both the nucleotide

and amino acid levels, occur at a relatively constant rate in equivalent genes in all organisms, thereby constituting a form of "molecular clock" from which one can statistically predict the relatedness of groups of organisms (2). As to which of the many statistical models constitutes the best analysis of DNA and protein sequence data is again the subject of an entertaining and sometimes acrimonious debate. The method employed in this analysis is the unweighted pair group method initially described by Sneath and Sokal (4). The unweighted pair group method aligns the amino acid sequences of multiple proteins in a pairwise fashion and groups the pairs with the highest degree of identity together on the same phylogenic branch (4). It is assumed that two related organisms will have fewer changed amino acids than a more distant relative.

Trypanosoma cruzi (*T. cruzi*), the causative agent of South American trypanosomiasis, is related to *L. donovani* and *T. brucei* both epidemiologically and morphologically. Epidemiologically, *T. cruzi*, like the other kinetoplastid parasites, has an insect vector, an animal reservoir, and causes a staggering number of infections, afflicting almost 50 million people annually. From a morphological standpoint *T. cruzi* has traditionally been classified as a trypanosome because of physical characteristics the initial infective stage shares with *T. brucei* and other trypanosomes.

From a phylogenic point of view it is generally accepted that *T. cruzi* is evolutionarily midway between *Leishmania* and *T. brucei*. However, the dilemma arises when one wishes to determine from which ancestral lineage *T. cruzi* diverged. Using the unweighted pair-group method and the AdoMetDC amino acid sequences it was predicted that *T. cruzi* diverged from the same common ancestor as *L. donovani* later than did *T. brucei*. This

conflicts somewhat with the only previously published molecular phylogenic study of these three organisms by Lake et al. which used the nucleotide sequence of the genes encoding mitochondrial rRNAs (5). Lake et al. arrived at the conclusion that *T. cruzi* is more related to *T. brucei* than to *L. donovani*.

There are several possible explanations for the discrepancy between the two studies. First, both models used partial *T. cruzi* sequences to calculate the evolutionary distances between the three organisms, because the entire sequence was not available. This increases greatly the stochastic error of the methods. Lake et al. stated that they could not determine at which point these three organisms diverged with statistical certainty, but from analysis of other indicators and clues, such as fossil records of the ancestral insect hosts of these organisms, reasoned that *T. cruzi* and *T. brucei* were the most related. Yet another possible reason for disagreement between these two phylogenies is that these statistical models assume a random mutation rate, which is probably not absolutely true for the parasite AdoMetDC enzymes. Because *Leishmania* and *T. cruzi* are both intracellular parasites, e.g., residing within the host macrophage, it is possible that their AdoMetDC enzymes were subject to convergent evolutionary forces, whereas *T. brucei*, as an extracellular blood-born parasite is subject to an entirely different environment.

The phylogenic model constructed using the AdoMetDC enzymes is, however, in good agreement with previously published phylogenies for other eukaryotes which were constructed using small subunit rRNA gene sequences (6), with the exception of the relative positions of the potato and yeast enzymes. Plants are slightly more related to mammals than are yeast (6) and this relationship is reversed in the AdoMetDC phylogenic model. A

likely explanation for this discrepancy is that the evolutionary distances between these two classes of organisms are too small to be measured with the "resolution" that the AdoMetDC sequences provide.

The ultimate contribution this phylogenic study therefore makes is to point out that the evolutionary relationships among these three parasites is not yet absolutely certain and that it deserves more investigation. One should remember that the predictions made by statistical models on a single gene or protein, as is the case here, are only probabilities and should be interpreted with care (2). As more genes are sequenced our view of evolution will become clearer just as the fit of a line to data becomes better the more independent experimental observations one has. Ideally this question could be resolved with the sequencing of the nuclear small subunit rRNA genes, which have been well characterized from many different organisms and are accepted as an impartial measure of evolutionary distances.

In conclusion, the study of the evolutionary relationships among the parasitic protozoa, as well as that of all organisms, has a value beyond that of pure scientific interest. Closely related organisms are expected to have similar biochemical traits and a fundamental knowledge of the evolutionary similarities and differences among human pathogens could ultimately point the way to the discovery of novel biochemical pathways and more effective chemotherapeutic targets (1).

REFERENCES

1. Pace NR, Olsen GJ, Woese CR. Ribosomal RNA Phylogeny and the Primary Lines of Evolutionary Descent. Cell 1986; 45:325-326.

2. Felsenstein J. Phylogenies from Molecular Sequences: Inference and Reliability. Annu. Rev. Genet. 1988; Suppl 22:521-565.

3. Vigilant L, Stoneking M, Harpending H, Hawkes K, Wilson A. African Populations and the Evolution of Human Mitochondrial DNA. Science 1991; 253:1503-1507.

4. Sneath PHA, Sokal RR. Unweighted pair-group method using arithmetic averages. Numerical Taxonomy, W H Freeman Co San Francisco 1973; Second edition:230-234.

5. Lake JA, Vidal F, Paulo CG, Carlos M, Simpson L. Evolution of parasitism: Kinetoplastid protozoan history reconstructed from mitochondrial rRNA gene sequences. Proc Natl Acad Sci USA 1988; 85:4779-4783.

6. Sogin ML, Elwood HJ, Gunderson JH. Evolutionary diversity of eukaryotic small-subunit rRNA genes. Proc Natl Acad Sci USA 1986; 83:1383-1387.

FIGURES

Figure 1. Phenogram of the results from pair-grouping of the nine reported AdoMetDC amino acid sequences plus the predicted amino acid sequence of the *T. cruzi* PCR product.

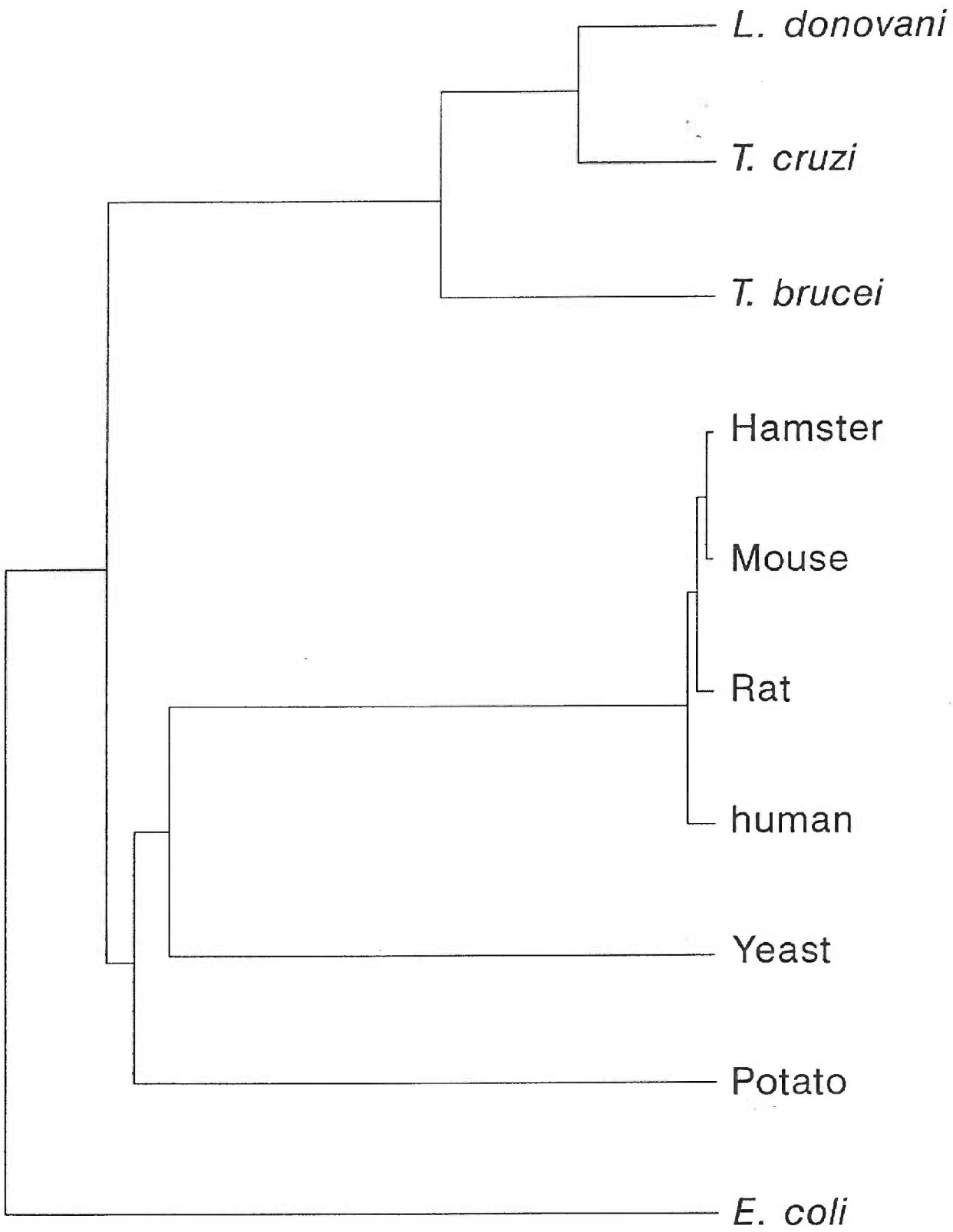

L. donovani
T. cruzi
T. brucei
Hamster
Mouse
Rat
human
Yeast
Potato
E. coli

Figure 2. Nucleotide and predicted amino acid sequence of protein coding region of the PCR product from a segment of the *T. cruzi AdoMetDC* gene and the PCR product of the spliced-leader PCR product derived from *T. cruzi* cDNA. The nucleotide sequence was determined in both directions as describe in the "Materials and Methods" section of the manuscript. An in-frame termination codon 5' to the predicted start site is underlined and the nucleotide that corresponds to the first nucleotide of the mature *AdoMetDC* transcript to which the mini-exon is *trans*-spliced is marked in bold with an asterisk.

```
                *
-87     GCGGAGTAGT TAGTGATTCT CGGAGTATGG CAATTACATA CATTTGGTTG CATTGCGTGT GCGGGTGGGT GTGTGTCTCG AGGTATC      -1

        ATG TTA AGC AAT AAG GAC CCT CTC TCT TTG ATG GCG ATG TGG GGT TCA GTG AAG GGC TAC GAC CCA AAT CAG GGG  75
        Met Leu Ser Asn Lys Asp Pro Leu Ser Leu Met Ala Met Trp Gly Ser Val Lys Gly Tyr Asp Pro Asn Gln Gly
        1

        GCC AGT TTT GAG GGG CCG GAA AAA CGT TTG GAG GTG ATC ATG CGT ATT ATT GAC GAG ACC CAC TCG GAG GGC CTC 150
        Ala Ser Phe Glu Gly Pro Glu Lys Arg Leu Glu Val Ile Met Arg Ile Ile Asp Glu Thr His Ser Glu Gly Leu
        26

        CAT GCC CTT GGA GAT GAG GTA TGG AAA GGT GTG GTG GGC TCG TTA AAT GCC CAA ATT GTA TCC AAA GAG AGT AAT 225
        His Ala Leu Gly Asp Glu Val Trp Lys Gly Val Val Gly Ser Leu Asn Ala Gln Ile Val Ser Lys Glu Ser Asn
        51

        AAT GAG TAT ATT CGC TCT TAT GTG CTA ACG GAA AGC TCA TTG TTT GTC ATG CGG GAC CGT ATC ATC CTG ATC ACA 300
        Asn Glu Tyr Ile Arg Ser Tyr Val Leu Thr Glu Ser Ser Leu Phe Val Met Arg Asp Arg Ile Ile Leu Ile Thr
        76

        TGT GGC ACG ACG ACT CTA CTT AAT GCC GTT CCT TTC GTT TTG GAC GCA GTT AGT GAT GTG CGA GGG GAG GTG GAA 375
        Cys Gly Thr Thr Thr Leu Leu Asn Ala Val Pro Phe Val Leu Asp Ala Val Ser Asp Val Arg Gly Glu Val Glu
        101

        TGG GTC TCT TTC ATG CAT AAG AAC TAC AGT TTT CCG TGG GAA CAG AAG GGA CCA CAT CTC TCC ATG GCA GAG GAA 450
        Trp Val Ser Phe Met His Lys Asn Tyr Ser Phe Pro Trp Glu Gln Lys Gly Pro His Leu Ser Met Ala Glu Glu
        126

        TTC                                                                                              453
        Phe
        151
```

Figure 3. Comparison of the amino acid sequences of the AdoMetDC proteins from *T. brucei*, *donovani*, and *T. cruzi*. The predicted amino acid sequence of the *L. donovani AdoMetDC* w compared sequentially with that of the *T. brucei AdoMetDC* and the *T. cruzi* PCR product. Verti lines (¦) denote identical amino acids and dots (.) signify conservative amino acid substitutio The sequence alignment was performed using the CLUSTAL V program of Higgins and Sharp described in the "Materials and Methods" section of the manuscript. Sequence similarities betwe the *T. brucei* and *T. cruzi* AdoMetDCs are marked with asterisks (*) for identical amino ami acids, and dots (.) for conservative substitutions, above the aligned sequences for comparison wi the other similarities marked between the respective sequences.

Sequence Alignment of the AdoMetDC Enzymes from T. cruzi, L. donovani, and T. brucei

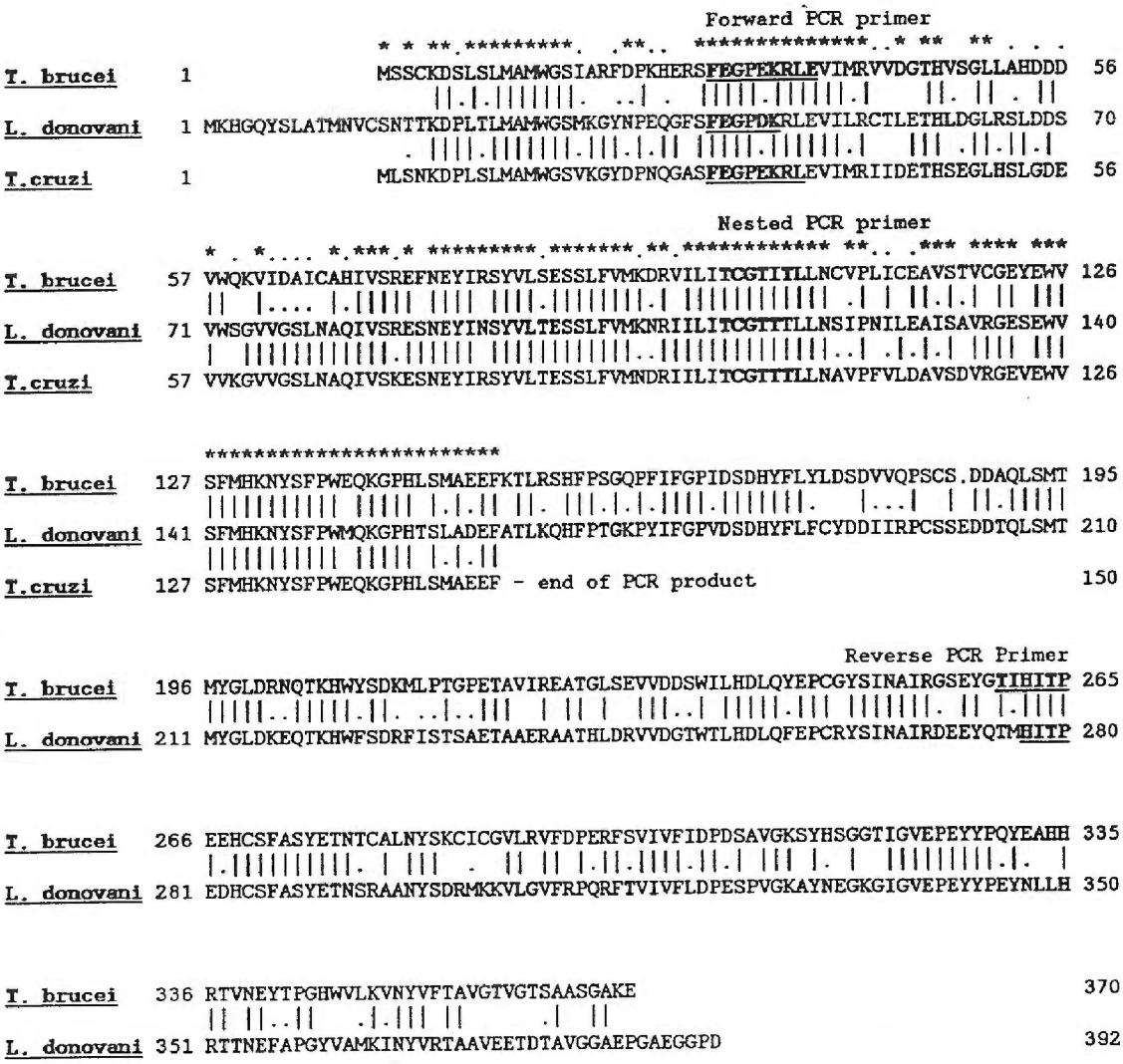


Figure 4. Alignment of eukaryotic AdoMetDCs with the *E. coli* AdoMetDC. The predicted amino acid sequences of the eukaryotic AdoMetDC proteins was compared aligned with the deduced amino acid sequence for the *E. coli* AdoMetDC according to the CLUSTAL V program as described in the Materials and Methods section. The purpose of the figure is to illustrate that there is essentially no homology between the bacterial and eukaryotic enzymes. Because the human enzyme is highly homologous to the other cloned mammalian genes, it is used as the representative mammalian sequence.


T.brucei M----------SSC----KDSLSLMAMWGSIARFDPKHERSFEGPEKRLE
L.donovani MKHGQYSLATMNVCSNTTKDPLTLMAMWGSMKGYNPEQGFSFEGPDKRLE
Human MEA----------AFEGTEKLLE
Yeast MTVTIKELTNHNYIDHELSATL----------DSTDAFEGPEKLLE
Potato MEMDL----------PVSAIGFEGFEKRLE
E.coli MKK----LKLHGFNNLTLSLSFCITDICTAKTAEERDGWIAWID-----E
T.brucei VIMR-----VVDGT---HVSGLLAHDDDVWQKVIDAICAHIVSREFNEYI
L.donovani VILR-----CTLET---HLDGLRSLDDSVWSGVVGSLNAQIVSRESNEYI
Human VWFSRWWPD--ANQ---GSGDLPTIPRSEWDILLKDVQCSIISVTKTDKQ
Yeast IWF---FPHKKSIT---TEKTLRNIGMDRWIEILKLVKCEVLSMKKTKEL
Potato ISFVE--PGLFADP---NGKGLRSLSKAQLDEILGPAECTIVDNLSNDYV
E.coli LWNANRLTEILSETCSIIGANILNIARQDWEPQGASV----------
T.brucei RSYVLSESSLFVMKDRVILITCGTITLLNCVPLICEAVSTV--CG--EYE
L.donovani NSYVLTESSLFVMKNRIILITCGTTTLLNSIPNILEAISAV--RG--ESE
Human EAYVLSESSMFVSKRRFILKTCGTTLLLKALVPLLKLARDY--SGFDSIQ
Yeast DAFLLSESSLFVFDHKLTMKTCGTTTTLFCLEKLFQIVEQELSWAFRTTQ
Potato DSYVLSESSLFVYSYKIIIKTCGTTKLLLAIPPILRLAETL--S--LKVQ
E.coli -TILVSEEPV---DPKLIDKTEHPGPLPETVVAHLDKSHI----------
T.brucei -------WVSFMHKNYS-FP-----WEQKGPHLSMAEEFKTLRSHF----
L.donovani -------WVSFMHKNYS-FP-----WMQKGPHTSLADEFATLKQHF----
Human -------SFFYSRKNFM-KP-----SHQGYPHRNFQEEIEFLNAIF----
Yeast GGKYKPFKVFYSRRCFRLFPCKQAAIHQAAIHQNWADEVDYLNKFFDN--
Potato -------DVRYTRGSFI-FP-----GAQSFPHRHFSEEVAVLDGYFGKLA
E.coli ----------C-----VHTYPESHPEGGLCTFRADIE---
T.brucei PSGQPFIFGPIDSD-HYFLYLDSD----VVQPSCS-DDAQLSMTMYGLDR
L.donovani PTGKPYIFGPVDSD-HYFLFCYDD----IIRPCSSEDDTQLSMTMYGLDK
Human PNGAGYCMGRMNSDCQY-LYTLDF----PESRVISQPDQTLEILMSELDP
Yeast --GKSYSVGRNDKSNHWNLYVTETDRSTPKQKEYIEDDETFEVLMTELDP
Potato AGSKAVIMGSPDKTQKWHVYSAS-----AGSVQSNDPVYTLEMCMTGLDR
E.coli ----------VSTCGVISPLKALNWLIHGLES
T.brucei NQTKHWYSDKMLPTGPETA----------VIREATGLSEV--------V
L.donovani EQTKHWFSDRFISTSAETA----------AERAATHLDRV--------V
Human AVMDQFY----MKDGVTAK----------DVTRESGIRDL--------I
Yeast ECASKFYCGPEASTTALVEPNEDKGHNLGYQMTKNTRLDEIEYVNSAQDS
Potato EKASVFY----KTEESSAA----------HMTVRSGIRKI--------L
E.coli DIVTI----------

```
T.brucei        DDSWILHDLQYEPCGYSINAIRGSE-YGTIHITPEEHCSFASYETNTCAL
L.donovani      DGTWTLHDLQFEPCRYSINAIRDEE-YQTMHITPEDHCSFASYETNSRAA
Human           PGS-VIDATMFNPCGYSMNGMKSDGTYWTIHITPEPEFSYVSFETNLSQT
Yeast           DLSFHHDAFAFTPCGYSSNMILAEKYYYTLHVTPEKGWSYASFESNIPVF
Potato          PKS-EICDFEFEPCGYSMNSIEGAAV-STIHITPEDGFTYASFES----V
E.coli          --DWRVRGFT--------RDIQGMKHFIDHEIQ-----SIQNFMSDDMLA
                                  .         ..       .  .. .

T.brucei        NYSKC-------ICGVLRVFDPERFSVIVFIDPDSAVGKSYHSGGTIGVE
L.donovani      NYSDR-------MKKVLGVFRPQRFTVIVFLDPESPVGKAYNEGKGIGVE
Human           SYDSYD-DLIRHILNVVEVFKPGKFVTTLFVNQSSK-----CRTVLA---
Yeast           DISQGKQD----NLDVLLVFQPREFSMTFFTKNYQN--QSFQKLLSIN--
Potato          GYNPKTMELGPLVERVLACFEPAEFSVALHADVATKLLERICSVDVKG--
E.coli          LYDMVD----------VNVWQENIFHTKMLLK-----------------
                                  .       *

T.brucei        PEYYPQYEAHHRTVNEYTPGHWVLKVNYVFTAV-----GTVGTS---AAS
L.donovani      PEYYPEYNLLHRTTNEFAPGYVAMKINYVRTAAVEETDTAVGGA-EPGAE
Human           ------------SPQKIEGGKRLDCQSAMFNDY----NFVFTSF---AKK
Yeast           -ESLPDYIKLDKIVYDLDD-YHLFYMKL----------------------
Potato          ------YSLAEWSPEEFGEGGSIVYQKFTRTPYCESPKSVLKGCWKEEEK
E.coli          -EFDLKHWMFHTKPEDLTDSERQEITAALWKE--------MREIWW-GRQ

T.brucei        GAKE
L.donovani      GGPD
Human           QQQQ
Yeast           QKKI
Potato          EGKE
E.coli          MPAV
```

Figure 5. Percentage identity between the amino acid sequences of the kinetoplastid, Yeast, Human, Potato, and *E. coli* AdoMetDC proteins. The percentages were calculated as described in the Materials and Methods section.

Comparison of AdoMetDC amino acid sequences (% identity)

	L.donovani	T.cruzi	Yeast	Human	Potato	E.coli
T.brucei	63%	70%	22%	27%	27%	11%
L.donovani	-	75%	22%	26%	28%	10%
T.cruzi	-	-	21%	27%	30%	7%
Yeast	-	-	-	34%	27%	11%
Human	-	-	-	-	33%	10%
Potato	-	-	-	-	-	8%

LIST OF ABBREVIATIONS

AdoMetDC	-	S-adenosylmethionine decarboxylase enzyme
AdoMetDC	-	*S-adenosylmethionine decarboxylase* gene
DDT	-	dichloro-diphenyl-dichloro-ethane
DFMO	-	α-difluoromethylornithine
HGPRT	-	Hypoxanthine-Guanine phosphoribosyltransferase
L. donovani	-	*Leishmania donovani*
MDL	-	Merryl Dow Labs
MDL 73811	-	5'-{[Z]-4-amino-2-butenyl]methylamino}-5'deoxyadensine
ODC	-	ornithine decarboxylase enzyme
pBAce	-	pB = Bluescript® phagemid, Ace = Alkaline phosphatase promoter-regulated cytosolic expression
pBlsd	-	pBace plasmid with the leishmanial *S-adenosylmethionine decarboxylase* gene insert
pBtsd	-	pBace plasmid with the trypanosomal *S-adenosylmethionine decarboxylase* gene insert
PEST	-	Proline, Glutamate, Serine, Threonine
T. brucei	-	*Trypanosoma brucei*
T. b. rhodesiense	-	*Trypanosoma brucei rhodesiense*
T. b. gambiense	-	*Trypanosoma brucei gambiense*
T. cruzi	-	*Trypanosoma cruzi*
rRNA	-	ribosomal RNA